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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

NEW OR NOTEWORTHY VAUCHERIAE FROM NEW ENGLAND SALT MARSHES

JOHN L. BLUM AND JOHN T. CONOVER

Dept. of Botany, University of Michigan, Ann Arbor, Mich., and Woods Hole Oceanographic Institution, Woods Hole, Mass.

The known *Vaucheria* flora of marine and brackish habitats in northeastern United States includes only four species, *V. thuretii* Woronin, *V. litorea* C. Agardh, *V. compacta* (Collins) Collins, and *V. piloboloides* Thuret. Apparently no additions have been made to this list since the publication of Taylor's Marine Algae of the Northeastern Coast of North America (1937). In the course of an ecological study of Great Pond, near Woods Hole, Massachusetts, winter and early spring collections by John T. Conover of salt-marsh and other coastal Vaucheriae have recently yielded three additional species which have not been previously reported from North America, and a fourth species which is new. Preserved material of these collections has been deposited in the Herbarium of the University of Michigan and in that of Professor William Randolph Taylor.

Section WORONINIA Solms-Laubach

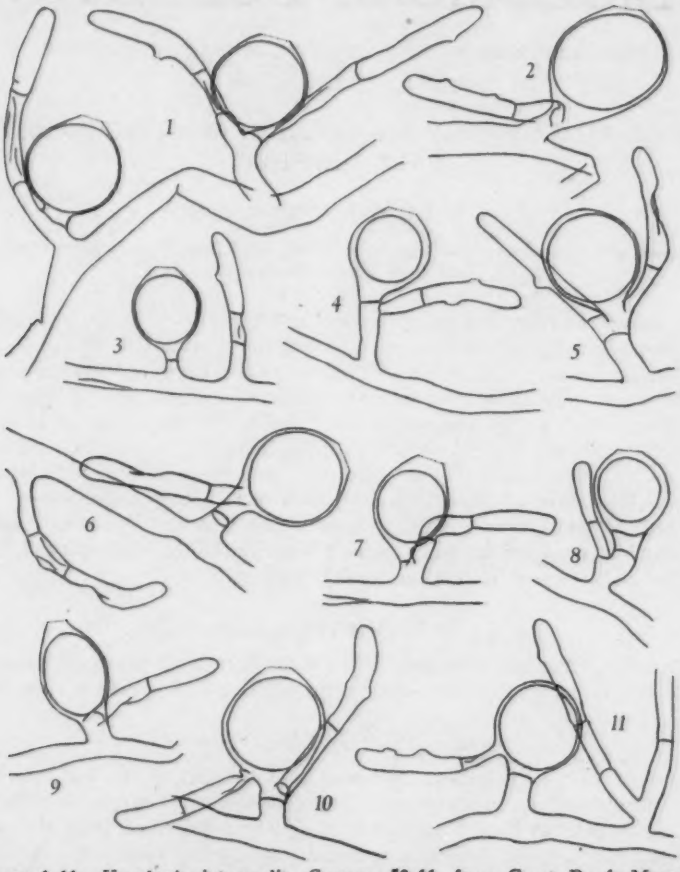
VAUCHERIA THURETII Woronin. *Specimens examined:* MASSACHUSETTS: East Shore Marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–6, Jan. 31, 1953.

Section CONTORTAE Dangeard

V. ARCASSONENSIS Dangeard. While most of the other species listed in this paper are known to be present in many localities on the coast of Europe, no collections of this species appear to have been made since it was described from the region of Arcachon, France. The American material was fruiting abundantly in April and early May, 1953. It does not seem to differ significantly in any way from the collection reported by Dangeard (1939). *Specimens examined:* MAINE: North shore, Harraseeket River estuary near South Freeport, Yarmouth Co., *Conover* 53–1, April 24, 1953. MASSACHUSETTS: Southeast shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–2, April 6, 1953; *idem, Conover* 53–3, May 19, 1953; West bank marsh, Great Pond, *Conover* 53–4, April 6, 1953; East shore marsh, Great Pond, *Conover* 53–5, May 11, 1953.

Section PILOBOLOIDEAE Walz em. Nordstedt

V. COMPACTA (Collins) Collins. This species was fruiting in the east shore marsh of Great Pond in January, 1953, mixed with *V. intermedia* Nordstedt and *V. thuretii*. It is reported by Taylor (1937) and has recently been identified by Christensen (1952) with the entity known in Europe as *V. sphaerospora* var. *dioica*



FIGURES 1–11. *Vaucheria intermedia, Conover* 53–11, from Great Pond, Massachusetts. Magnification approximately 160 ×. All figures drawn with the aid of a Spencer camera lucida.

Rosenvinge. *Specimens examined:* MASSACHUSETTS: East shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–6, Jan. 31, 1953.

V. CORONATA Nordstedt. This well-known European species has also been reported from Greenland (Collins, 1909). The material from Great Pond was fruit-

ing abundantly from February through April, 1953, and was more plentiful here than any other species. The American specimens agree in all details with Nordstedt's description. *Specimens examined:* MAINE: North shore, Harraseeket River estuary, near South Freeport, *Conover* 53–7, April 24, 1953. MASSACHUSETTS: East shore marsh, Great Pond, near Falmouth, Barnstable Co., *Conover* 53–8, March 22, 1953; Southeast shore marsh, Great Pond, *Conover* 53–9, April 6, 1953; West bank marsh, Great Pond, *Conover* 53–10, April 6, 1953; Southwest shore of Perch Pond (northwest arm of Great Pond estuary), *Conover* 53–16, May 11, 1953.

V. INTERMEDIA Nordstedt (Figs. 1–11). This species is likewise well-known in Europe. It is characterized by relatively small dimensions throughout. The fruiting branch is short and in the American material bears one oogonium and usually one or two antheridia. The oogonium is more or less erect and opens by a wide, circular pore at its upper end. The oospore is usually spherical or sub-spherical, although it is sometimes ovoid (Fig. 9). The antheridium is cylindric and straight and opens by usually one to three lateral pores at the end of very short lateral tubes. It is always subtended by what appears to be a thin-walled hyaline supporting cell, but is probably only an empty space.[1] These two units, together with any portion of the thallus which may be in linear connection with them, can be called an antheridial filament. When borne on a bisexual fruiting branch, the antheridia are terminal as in the Racemose Vaucheriae; presumably they are formed well before the oogonia, a point which cannot be checked because of the absence of developmental stages in this material. The oogonium may develop from a lateral outgrowth of the lower portion of the fruiting branch, which is continuous with the vegetative filament; in this case the oogonium soon becomes cut off by a cross wall from the adjacent portion of the fruiting branch. The oogonium may, however, develop as an intercalary cell in a linear series which has the lower portion of the fruiting branch as its base and the antheridium at its distal end. Due to the unilateral, outward growth of the oogonium, however, the antheridium always appears to be in a lateral position when the oogonium is mature.

There are essentially four different ways in which the antheridial filament may be attached with reference to the oogonia:

1. On a separate (antheridial) branch of a vegetative filament, usually near an oogonial or bisexual fruiting branch (Figs. 3, 6).
2. Terminal on a bisexual fruiting branch, but apparently borne on a separate (antheridial) branch of the basal portion of the fruiting branch (Figs. 4, 8).
3. Terminal on a bisexual fruiting branch, but apparently subtending the oogonium, attached to its cylindric basal portion (Figs. 5, 6).
4. Terminal on a bisexual fruiting branch, but apparently a lateral offshoot of, or even surmounting, the oogonium, as a consequence of the lateral enlargement of the latter about a point more or less within the original axis of the fruiting branch (Figs. 10, 11).

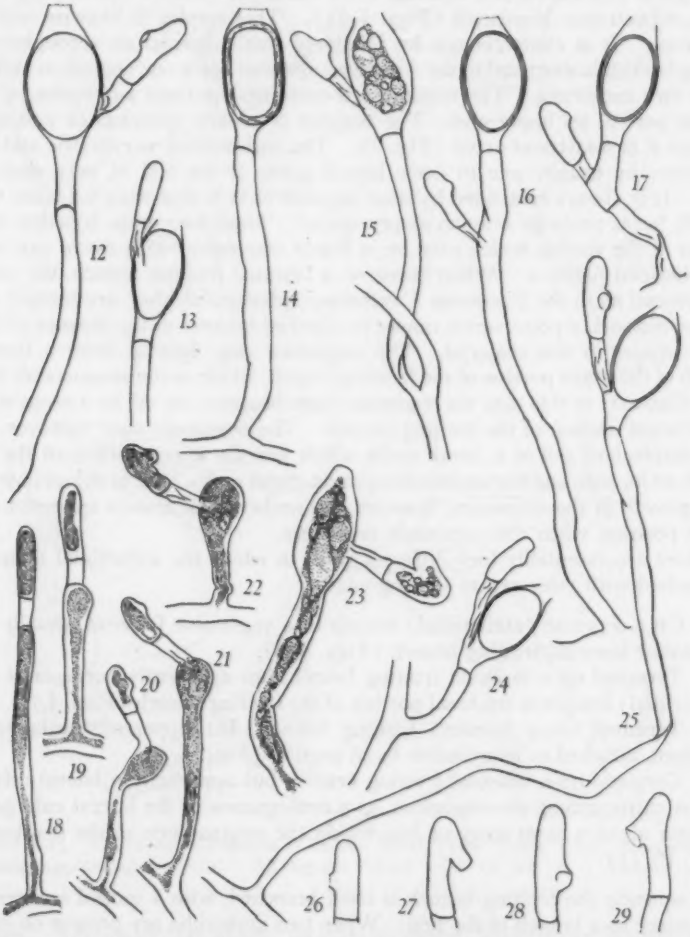
Frequently the fruiting branch is itself branched, with a second antheridial filament borne as a branch of the first. When two antheridia are present on one fruiting branch, the oogonium generally lies somewhere between them (Figs. 1, 5, 10).

Vegetative diameter of the American material ranges from 14 μ to 35(–51) μ.

[1] Cf. Dangeard, 1939, with reference to *V. litorea*.

Antheridia measure 12–19(–34) × (51–)70–110(–124)μ. Oogonia measure 51–91 × 78–112 μ. The oospores, usually spherical, measure 52–94 μ in diameter. The length of the basal part of the fruiting branch, measured from the vegetative filament to the oogonial cross wall, varies from 11–76 μ. These measurements are in general agreement with those given by Nordstedt (1879) and Dangeard (1939).

This species was fruiting abundantly in Great Pond in January, 1953, mixed with *V. thuretii*, *V. compacta*, and *V. minuta* sp. nov. *Specimens examined:*



FIGURES 12–29. *Vaucheria minuta* sp. nov. *Conover* 53-12, type, from Great Pond, Massachusetts. Figures 12–17, 24, 25, mature or nearly mature bisexual fruiting branches. Figures 18–23, immature fruiting branches. Figures 26–29, antheridia. Magnification approximately 160 ×. Figures 12–25 drawn with the aid of a Spencer camera lucida.

MASSACHUSETTS: East shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–11, Jan. 31, 1953.

Vaucheria minuta sp. nov. (Figs. 12–29).

Filamenta vegetativa 8.5–15(–17)μ diam.; oogonia antheridiaque in ramo fructifero longo portata, antheridiis terminalibus, per spatium vacuum celluliforme subtensis, oogoniis intercalaribus proxime infra hoc spatium enascentibus, aut oogoniis terminalibus, antheridiis nullis; antheridium ad oogonii maturi latus basimve plerumque affixum; rami fructiferi 195–820μ long., antheridia cylindrica, 13.5–17 × 47–64μ, e poro laterali unico, oogonium aspiciente, aperienta; oogonium symmetricale, ovatum ad cylindricum, 51–54(–64) × 102–125(–170)μ, e poro terminali erecto lato aperiens; oospora ovata ad cylindricam, 50–53(–62) × 68–92μ, membrana oosporae maturae satis crassa.

Vegetative filaments 8.5–15(–17)μ in diameter; oogonia and antheridia borne on a long fruiting branch, the antheridia terminal, subtended by an empty cell-like space, the oogonia intercalary and arising immediately below this space; or the oogonia terminal and antheridia absent; at maturity of the oogonium, the antheridium is usually attached to the side or base of the oogonium; fruiting branches 195–820μ in length; antheridia cylindrical, 13.5–17 × 47–64μ, opening by a single lateral pore which faces the oogonium; oogonium symmetrical, ovoid to cylindric, 51–54 (–64) × 102–125(–170)μ, opening by a wide, erect terminal pore; oospore ovoid to cylindric, 50–53(–62) × 68–92μ, with a relatively thick wall at maturity.

This species is distinguished from *V. intermedia* (1) by the extremely small size of its vegetative filaments, (2) by its relatively shorter antheridia, each bearing a single pore, (3) by the characteristic ovoid to cylindric oospores, which contrast markedly with the spherical oospores of *V. intermedia*, and (4) by the very much elongated fruiting branches, quite unlike the short branches of *V. intermedia*.

It is, however, clearly related to the latter species as well as to *V. sphaerospora* Nordstedt since these are the only species known to possess an antheridial filament which may surmount and have its point of attachment directly on the oogonium. Because of this peculiarity of the antheridial filament, which appears frequently as if it were an epiphyte on the oogonium, these three species are to be considered as set somewhat apart from the remaining species of the Section Piloboloideae.

The development of the antheridium and oogonium has been studied in preserved material by the comparative observation of immature fruiting branches of different ages (Figs. 18–23). It appears likely that the antheridium is always terminal on a bisexual fruiting branch, although this point must remain in doubt until development of the fruiting branch can be followed in living material. Several fruiting branches were found in which the filament immediately below the antheridial supporting cell or space was only slightly enlarged (Figs. 19–21). Later stages show the antheridial filament clearly pushed aside and tipped from its apical, erect position by the unilateral and upward enlargement of the young oogonium (Figs. 22–23). A similar series of developing fruiting branches has been illustrated for *V. sphaerospora* by Nordstedt (1879). Filaments lacking an enlargement, such as the one shown in Figure 18, are observed rather commonly; actually there is no assurance that these are destined to form bisexual fruiting branches—they may merely represent antheridial branches which will never form an oogonium.

V. minuta is distinctive and unlike other Vaucheriae in at least two ways: (1) its vegetative filaments are smaller in diameter than those of any other known species,

and (2) it has cylindric antheridia which open by a single lateral pore borne at the end of a short tube. Descriptions of all of the other members of the Piloboloideae indicate that the antheridia have a variable number of pores, usually 1–2 or 1–3. In *V. minuta* the single pore with its tube usually faces the oogonium and frequently is directed somewhat toward the base of the antheridial filament (Figs. 23, 27, 29). Occasionally this lateral tube is so well developed as to give the whole antheridium the appearance of being reflexed (Fig. 23). As in *V. intermedia,* the antheridium is subtended by a sterile supporting cell or empty space which is thin walled and lacks protoplasm. Likewise as in *V. intermedia,* the antheridial filament is highly variable in position, and may be found in the same series of situations listed above for *V. intermedia;* however, in this connection it may be mentioned that no unquestioned short antheridial branches have been found on vegetative filaments of *V. minuta* (position 1). All of the isolated "antheridial" branches are hence open to the suspicion of being immature fruiting branches as yet lacking the oogonial enlargement.

Fruiting branches which lack antheridia (Fig. 14) are common. It is impossible to determine from preserved material whether the "oogonia" formed on these structures contain oospores or parthenospores. Usually these spores are more distinctly cylindrical, and thicker walled, than are the undoubted oospores borne on bisexual fruiting branches.

V. minuta was found fruiting in Great Pond in March and May, 1953, and sparsely fruiting in the Harraseeket River estuary in April. Its habitats range from submerged areas of relatively high salinity (29.5–30.2 parts per thousand) to emergent situations subject to wide range in salt concentration. At the time of fruiting, the algae were covered by water of salinity ranging from 26.0 to 28.0 parts per thousand. *Specimens examined:* MAINE: North shore, Harraseeket River estuary, near South Freeport, Yarmouth Co., *Conover* 53–17, April 24, 1953. MASSACHUSETTS: TYPE in Herbarium of the University of Michigan; east shore marsh, Great Pond, Falmouth, Barnstable Co., *Conover* 53–12, March 22, 1953; *idem, Conover* 53–14, Jan. 31, 1953; *idem, Conover* 53–15, May 11, 1953.

ECOLOGICAL REMARKS

The Vaucheria species here discussed have been collected near spring and neap high tide levels and appear to be confined to this relatively narrow zone on the borders of estuaries or in well drained brackish marshes which are arms of the sea at high tide. These algae regularly grow as a mat of 2–4 *Vaucheria* species mingled with Myxophyceae and Chlorophyceae of brackish water. This mat commonly occurs in scattered, moderately dense to sparse stands of *Juncus, Spartina,* or various other graminioids, covering their rhizomes and the intervening soil surface over an area which may be as much as twenty-four square meters in extent. When the water from the estuary does cover the algal mats, it ranges from 12.0 to 27.0 parts per thousand in salinity. Due to strong onshore winds in late fall of 1952 and early spring of 1953, however, the *Vaucheria* vegetation was covered continuously at these times for a period of several days by water of somewhat higher salinity (26.0–28.0 parts per thousand). The mats of *Vaucheria* may be covered by brackish water at times of spring and neap tides, but the water level of the usual diurnal tides apparently does not reach them. During such periods when the algal zone is emergent, the *Vaucheria* mats are subject to fresh runoff. They are hence exposed regularly to

wide variations in salinity, from that of fresh water to very nearly the concentration of the sea.

Partial shade seems to be necessary for the growth of these *Vaucheria* species. Although they grow commonly over sandy to peaty soil at the base of graminioid plants, they are not found where these phanerogams grow densely and shade is heavy. Neither are they found in unshaded areas.

While the seasons at which these species were found in fruit varied from species to species, none were found in fruit at temperatures above 19.0 degrees Centigrade. Temperatures which favor fruiting range from 0.0 to 18.5. No fruiting *Vaucheria* was found in any of the numerous collections made from the same meter square quadrats in the warmer months from late May through November.

In view of the relative thoroughness with which the Woods Hole area has been explored by phycologists in summer, it would appear that most of these species have escaped detection because of a fruiting period which is confined to the winter months. The near-absence of fruiting material in all *Vaucheria* collections made in Great Pond in the period from May through July, 1953 continues to bear out this supposition.

The authors wish to thank Professor William Randolph Taylor for the advice he has given during the progress of this work and for his critical reading of the manuscript.

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CUTANEOUS AND TRACHEAL RESPIRATION IN THE PHORMIA LARVA

JOHN B. BUCK AND MARGARET L. KEISTER

Laboratory of Physical Biology, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland

The larvae of most calypterate flies, of which *Phormia regina* Meig. is an example, have a relatively simple amphipneustic tracheal system consisting basically of a pair of main longitudinal trunks opening to the exterior through one pair of anterior and one pair of posterior spiracles (Fig. 1A, 1B). The anterior and posterior spiracles are strikingly different in structure, but no measurements of their relative importance in the over-all gas exchange of the larva have been reported previously. Similarly, except for the work of Fraenkel and Herford (1938) on *Calliphora*, there are practically no quantitative data on the relative magnitudes of oxygen uptake through the skin and by way of the tracheal system. The *Phormia* larva has proved to be excellent material on which to investigate these problems, particularly because it has remarkable tolerance to radical experimentation: for example, it will live for many hours with either or both ends of the tracheal system closed off by ligatures. Some of the results obtained have been published in abstract (Buck and Keister, 1950a, 1950b; 1953).

The assistance of Mary Ann Dawson in part of this work is acknowledged with pleasure.

Material and Methods

Third instars of *Phormia regina* were collected at about the time of emptying of the digestive tract preparatory to pupation (about 5 days after egg laying, at 25° C.). Culture methods, techniques for holding and ligating the larvae and procedures for stimulating respiration with DDT have been described previously (Buck, Keister and Posner, 1952). In all cases "ligated" refers to larvae with ligatures cutting off one or both pairs of spiracles, whereas "deganglionated" refers to larvae with a ligature just posterior to the brain (which also, of course, closes the tracheae at that point). "Hypoxia" is used to mean a condition in which some aerobic respiration is occurring, but in which oxygen uptake is limited by pO_2.

In applying ligatures there is no direct test of whether the tracheae are actually pinched completely shut, and since the logic of the whole investigation depended on this being true, the ligatures were made tighter than was probably necessary. As previously reported, this not infrequently resulted in one or both of the tracheal trunks actually being severed, particularly in ligating off the anterior spiracles. Less trouble was experienced with deganglionations, since the ligature needed to be only tight enough to prevent leakage when the head end was removed. Whenever blood entered a cut trachea the larva was discarded. Usually, however, the trachea was quickly sealed off at the break point by a small melanotic plug or cap formed at the interface between blood and intratracheal gas. Since the trachea remained *in situ*

and since there was no other visible internal damage—*e.g.*, to the gut—such larvae appeared to be physiologically equivalent to larvae in which the tracheae were not cut, and might even be considered to have the advantage of giving visible proof that no gas could enter the tracheal system through the spiracle in question. Since also, in an extensive series of tests, larvae with cut trunks behaved and respired indistinguishably from those with intact ligated trunks—in sharp distinction to the moribund and severely hypoxic larvae with flooded tracheae—such larvae, representing about 25% of the anteriorly ligated, 10% of the posteriorly ligated, and 10% of the deganglionated animals, were used in the respirometry.

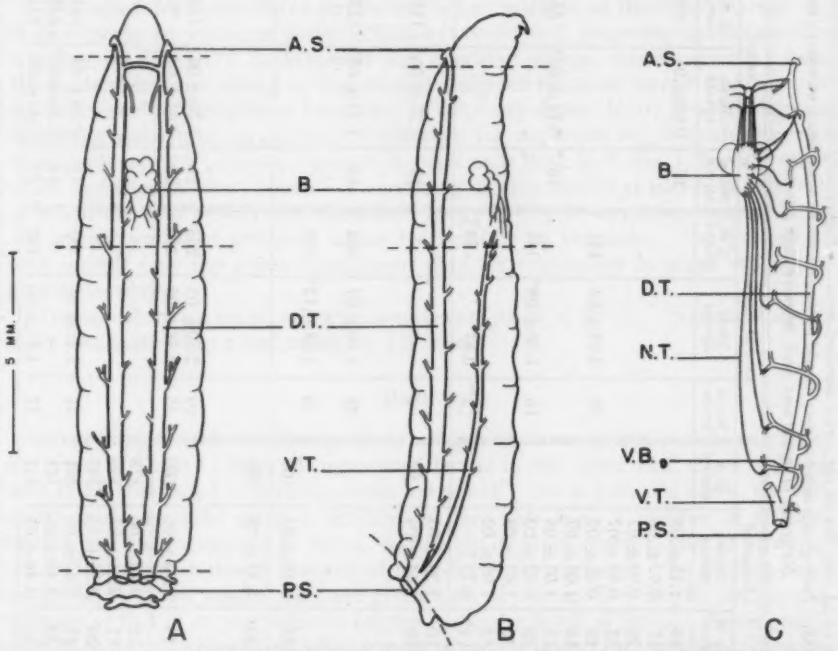

FIGURE 1. A and B, dorsal and lateral diagrammatic views of tracheal system of third stage larva of *Phormia regina* Meig. C, diagram (at slightly less magnification than A and B) of ventral view of neural tracheae (solid black), the dorsal trunk being displaced to the apparent right. A.S. = anterior spiracle; B = brain; D.T. = dorsal trunk; N.T. = neural trachea; P.S. = posterior spiracle; V.B. = ventral branch of dorsal trunk; V.T. = visceral trunk. Dotted lines indicate positions of ligatures.

Improvements made in our technique for administering "light" doses of DDT (Buck, Keister and Posner, 1952) involved using only the minimum clamp pressure necessary to hold the larva (which eliminated leakage due to back pressure), injecting always through the center slit of the left posterior spiracle, and selecting only larvae having DDT solution filling at least the left medial neural trachea (Fig. 1C). With the dose used (approximately 0.6 μL per 60 mg. larva, corresponding to 50 mg./kg. live weight) all larvae were incapable of locomotion at 3½ and at 8

TABLE I

Mean oxygen uptake of Phormia larvae in μL/mg. live wt./hr., with standard errors. Figures in brackets are for Calliphora at 27° from Fraenkel and Herford, 1938. Inter-group comparisons should be made between same batch numbers (see text). No group of larvae was used for more than one run.

		Batch	Unligated			Anterior ligation			Posterior ligation			Double ligation		
			No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control
Unpoisoned	Air	292	18	1.15±.03					17	0.69±.01	60			
		373	17	0.92±.08								17	0.09±.003	10
		377	20	0.99±.02								19	0.09±.005	9
		379	22	0.95±.02										
		383	18	0.67±.02		29	0.95±.04	141						
		384	18	1.04±.07										
		385	17	1.07±.06					16	0.71±.03*	66			
		387	20	0.92±.02		16	1.30±.08*	141						
		388	26	1.07±.03										
		446	54	1.09±.02										
			[4**	0.72]		[5**	0.67	93]				[5**	0.15	21]
	Oxygen	273–4	10	1.24±.05										
		292	19	1.11±.04	97				17	1.27±.06	110			
		373										16	0.37±.02	39
		377										21	0.36±.01	36
		383				28	1.10±.07	164	18	1.32±.05*	123			
		385												
		387				16	1.48±.12*	161						
		388	25	1.10±.02	103							[5**	0.38]	
Poisoned	Air	273–4	20	2.55±.08	206									
		373										18	0.09±.004	10
		377										20	0.10±.01	10
		379				19	2.50±.05	263	21	1.20±.04	126			
		446	33	2.74±.06	250	35	2.38±.05	218	23	1.04±.02	96			
	Oxygen	273–4	18	3.45±.06	278									
		373	17	3.21±.05	349							18	0.35±.01	38
		377	20	3.58±.12	362							22	0.34±.01	34
		379	21	3.43±.06	361	21	3.37±.08	355	21	2.39±.04	251			
		384	30	3.48±.04	341				22	2.37±.05*	228			
		446	32	3.46±.07	317	37	3.21±.06	294	27	2.42±.03	222			

* Another run also made, with almost identical results, on larvae ligated immediately after poisoning and compared with separate contemporaneous controls. ** Indicates number of flasks, each containing about 10 larvae, run by Fraenkel and Herford.

hours after poisoning (tested on wet filter paper) but were often capable of locomoting at 24 hours, and were usually successful in forming apparently normal puparia (although few succeeded in emerging as adult flies).

Oxygen uptake was measured individually in 15-ml. Warburg flasks, using cylindrical wire screens to keep the control larvae out of the KOH, as described for adult flies by Buck and Keister (1949). The oxygen uptakes given in the table are means over a two-hour plateau period, usually between 5½ and 8 hours after the start of poisoning. Manometers were shaken at about 110 cycles per minute to aid temperature control.

The schedule of procedures carried out by two workers on the 40-odd larvae used in an experiment averaged about as follows: hours 0–1, poisoning by intratracheal injection of 5% DDT in kerosene and checking dosage microscopically; hours 3½–4, weighing and setting up control and unligated poisoned larvae (the latter being first tested for inability to locomote) in Warburg flasks; hours 3½–4½, ligating, inspecting and setting up control and poisoned larvae; hours 4½–5, flushing certain flasks with oxygen (3½–4 L. passed through each flask in 5 min.), leaving others in air, and equilibration; hours 5–8, recording oxygen uptake at half-hour intervals, of which the first period was discarded; hours 8–8½, re-inspecting ligated larvae and testing unligated poisoned larvae for inability to locomote. The present results include only gas phase respiration: data for respiration in water will be reported elsewhere.

Unless otherwise noted, all experiments were made at 25° C. Statistical significance was assessed by t test, using the 2% level.

Results

Oxygen uptakes from gaseous air or oxygen of larvae under various conditions are given in Table I; those of unpoisoned larvae in the upper half, those of larvae with DDT-stimulated respiration in the lower half. As in previous work, the variability in the material made it desirable to give data from a number of different batches for each category of larva, each with its own control, and to draw all conclusions from statistical analysis of intra-batch comparisons. To facilitate such comparisons, mean uptake rates are given both in μL/mg. live wt./hr. and as percentages of that of normal controls for the particular batch of larvae. Some groups seem to be more homogeneous when compared on one basis (*e.g.*, unligated, poisoned in oxygen) and some on the other (*e.g.*, anterior ligated, unpoisoned).

A. Cutaneous respiration

In gaseous air the oxygen uptake of doubly ligated larvae was approximately 10% of that in unligated controls (Table I). It is thus clear that larvae in which spiracular pathways of oxygen entry have been eliminated must become severely hypoxic.

In doubly ligated larvae poisoned intratracheally with DDT, oxygen uptake from air (10%) was no higher than that of doubly ligated unpoisoned larvae, in striking contrast to the great increase seen in poisoned unligated or once-ligated larvae in gaseous air or oxygen (Buck, Keister and Posner, 1952, and below). This, therefore, seems to indicate that, regardless of the potential metabolic rate, the maximum

it indicates that even the stimulation due to the ligature makes the larva mildly hypoxic, and that the posterior spiracles alone, though adequate for control respiration, are only just so.

The metabolic stimulation due to DDT is apparently so great that even with four times as much oxygen available neither pair of spiracles by itself is adequate.[1] As a matter of fact, even the unligated poisoned larvae in oxygen might prove to be hypoxic—i.e., capable of a higher rate of oxygen uptake—if tested in pure oxygen at greater than atmospheric pressure.

It is understandable that the absolute respiratory rates of the poisoned larvae, both in air and in oxygen, should be in the order unligated > anteriorly ligated > posteriorly ligated > doubly ligated, since this reflects the order of the "capacities" of the available pathways of oxygen entry. On the other hand, the fact that the *percentage* increases in uptake shown by the various groups in oxygen as compared with air are in the reverse order in both unpoisoned and poisoned larvae is consistent with the expected order of severity of hypoxia. Thus the most hypoxic groups, the doubly ligated larvae, take up four times as much oxygen from pure oxygen as batchmates do from air, whereas the poisoned unligated larvae, which have all their normal respiratory avenues intact, show only about a 30% increase. The non-linearity of the *increase* in respiration with five-fold increase in pO_2 is well shown in Figure 2. Though unexplained, the curious inability of the poisoned singly ligated larvae to take up as much oxygen from oxygen as the unligated poisoned larvae (*i.e.*, to fully eliminate hypoxia) deserves attention.

Discussion

Other things being equal, it might be expected that the oxygen uptakes of the 60-mg. *Phormia* larva and the morphologically similar 80-mg. larva of *Calliphora* would be of the same order of magnitude. As shown in Table I this expectation is borne out fairly well for most of the comparable types of experiment in spite of the considerable differences in experimental techniques. One minor discrepancy is that Fraenkel and Herford (1938) reported uptakes from air slightly below normal in *Calliphora* larvae with the anterior spiracles ligated off, whereas we observed in *Phormia* an uptake about 40% above the control, which we attribute to the greater activity caused by the irritation of the ligature. Another difference is in the apparent proportion which cutaneous respiration forms of the total oxygen uptake from air (21% in *Calliphora;* 10% in *Phormia*), which is especially surprising in view of the fact that in *Calliphora* respiration was measured on samples of 10 larvae simultaneously, where constant mutual contact stimulation might have engendered an artificially high level of control activity. Possibly the difference is due to Fraenkel and Herford's having followed respiration for only an hour, a period which, in *Phormia,* is marked by violent struggling of the doubly ligated larvae, whereas all our calculations represent the means of the uptakes over a two-hour period several hours after the start of the experiment. However, there may well be a real interspecific difference in cutaneous permeability to oxygen, particularly in view of the

[1] In batch 379 the O_2 uptake of unligated poisoned larvae in oxygen was not significantly higher than that of the anteriorly ligated poisoned larvae in oxygen (3.43 vs. 3.37), but the difference between the corresponding groups in batch 446 is so great that a highly significant difference ($p = < 0.01$) is obtained even if the two batches are lumped. It is evident that the anteriorly ligated larvae in oxygen are able to take in almost enough oxygen to avoid hypoxia.

difference in thickness of the cuticles and in reaction to paraffin-alcohol mixtures (Fraenkel, personal communication).

Fraenkel and Herford attempted to circumvent the effect of voluntary activity by measurements on deganglionated larvae. These respired at a rate which was about half that of normal controls and which was assumed to be the basal rate. They found that the oxygen uptake of deganglionated larvae with the hind spiracles also ligated off was about one quarter of that of deganglionated larvae with functional rear spiracles (thus one-eighth that of normal larvae). As already pointed out, this would actually correspond, in normal (non-hypoxic) controls, to much less than an eighth (12%) of the total uptake. By indirect methods Fraenkel and Herford estimated that the true cutaneous respiration is about 10% of the basal (hence 5% of the normal control). Buck, Keister and Posner (1952) found a drop of 32% in the oxygen uptake of deganglionated *Phormia* larvae at 25° in one batch, and a nonsignificant drop of 7% in another. In check experiments for the present paper, each involving 40–50 larvae, we found a nonsignificant drop of 4% and a nonsignificant increase of 3% at 25° and a significant drop of 16% at 30°. Since Fraenkel and Herford did not check their larvae for leakage of blood into the tracheae, which occurs rather frequently in *Phormia,* it is possible that the presumed basal rate in *Calliphora* is too low because it represents an average of the uptakes of larvae with flooded tracheae (and hence respiring at the very low rate of twice-ligated larvae), and others with rates close to that of unligated controls.

The increased cutaneous respiration in doubly ligated larvae in oxygen (Table I) is the expected response of these severely hypoxic animals to increased environmental pO_2. The unchanged cutaneous respiration after poisoning is therefore especially significant, not only because it shows that DDT has not affected cutaneous permeability to oxygen, but because it indicates that DDT has not significantly affected the oxygen gradient between environment and tissues (assuming that static diffusion is the mechanism of gas transport across the integument). The simplest explanation of the non-effect of DDT is that the internal pO_2 is already zero even in the unpoisoned doubly ligated larva, and oxygen is hence already penetrating the integument at its maximum rate for the oxygen gradient of about 136 mm. Hg, which obtains in the Warburg flask at 25°. This is what Fraenkel and Herford (1938) had postulated for doubly ligated *Calliphora* larvae, on the basis of their work with varied pO_2. As a matter of fact, the equality of oxygen uptake in unpoisoned and poisoned doubly ligated *Phormia* larvae in *oxygen* (Table I) shows that even with a trans-cutaneous gradient of 737 mm. the tissue pO_2 is essentially zero, indicating *a fortiori* that it must be zero in such larvae respiring air.

By making use of the oxygen uptakes measured at the known gradients, the permeability of the integument to oxygen can be calculated. Thus, using the mean total skin area of 8 doubly ligated 60-mg. larvae (85 $mm.^2$), the permeability for air respiration (0.09 μL/mg. live wt./hr.) is 0.0056 $\mu L/mm.^2/min./atm.$, and the value for oxygen 0.0042. A. G. Richards (personal communication) found good agreement between cutaneous permeability calculated from Warburg measurements on two doubly ligated larvae of *Sarcophaga bullata* in O_2 (the absolute values being about the same as in *Phormia*) and that calculated from oxygen electrode measurements on the isolated cuticles of the same larvae, using O_2-saturated water on one side and N_2-saturated water on the other. This supports the suggestion that the

tissue pO_2 in doubly ligated larvae quickly falls to zero and that the recorded rate for cuticular penetration is indeed maximal.

Assuming that neither DDT nor oxygen causes a physical change in the pathways of oxygen entry, it seems reasonable to postulate that if oxygen uptake via the three possible routes (skin, anterior spiracles, posterior spiracles) could be measured in larvae in which the internal pO_2 was the same, the ratio skin : ant. : post. would be the same as in the normal larva even though the internal pO_2 in the experimental animals was different from the normal. In such circumstances one would expect the following arithmetical relation to hold: total uptake (*i.e.*, unligated larva) = (cutaneous uptake) + (uptake of anteriorly ligated larva minus cutaneous uptake) + (uptake of posteriorly ligated larva minus cutaneous uptake). In the present series of experiments it seems clear that a strict quantitative separation of the avenues of oxygen entry is not possible, because the ligating which is necessary to separate the three pathways causes different degrees of hypoxia and hence modifies the inwardly directed oxygen gradients. This is indicated, for example, by the relatively poor fit obtained in substituting, in the above equation, the oxygen uptakes for the most severely hypoxic groups (poisoned, in air) of batch 446, using any value up to 0.1 for the small cutaneous correction factors (x_1, x_2, x_3):

$$2.74 = x_1 + (2.36 - x_2) + (1.04 - x_3).$$

A rigorous budget could presumably be made out either (1) by subjecting the four classes of larvae to reduced pO_2 and comparing their oxygen uptakes at the highest respective pO_2s at which DDT had no stimulating effect (*i.e.*, reducing all groups to zero internal pO_2) or (2) by comparing the uptake from air of each group at the highest temperature at which flushing with pure oxygen had no effect (*i.e.*, lowering, in most cases, the temperature until the metabolic requirements could be met by the amount of oxygen which could enter through the restricted avenues). Experiments along both the lines suggested above are under way, but for present purposes useful approximations can be derived from the data already available (Table I). For example, if we average the uptakes of the two batches in each of the ligated poisoned groups in air we get, neglecting the corrections for cutaneous uptake, a cutaneous : anterior : posterior ratio of 0.095 : 1.12 : 2.44, or approximately 1 : 12 : 26. As already discussed, we know that the cutaneous contribution must normally be much less than in doubly ligated larvae, but this at least shows us at once that it cannot be *more* than 1/39 (2.5%) of the total. Furthermore, although the indicated uptake through the anterior spiracles alone should be proportionately too high, both because the posteriorly ligated animal is more hypoxic than the anteriorly ligated and because of the irritating effect of the ligature, an A : P ratio of 12 : 26 or 1 : 2 is probably roughly correct. This is indicated by the facts (1) that the A : P ratio is approximately the same in both unpoisoned and poisoned larvae in air; (2) that the percentage increase in respiration in oxygen as compared with air in the poisoned anteriorly ligated larvae (130%) is close enough to that in the poisoned posteriorly ligated larvae (210%) to indicate that their hypoxias are at least of the same order of magnitude; and (3) because the observed uptake through the anterior spiracles is undoubtedly lowered, as compared with normal, because the ligature cuts off the visceral tracheal trunks from the main longitudinal trunks at the same time it closes the posterior spiracles (Fig. 1). Furthermore, in experiments in which oxygen intakes by the two ends of the body were measured separately in

normal control larvae stuck through a rubber membrane, an A : P ratio of approximately 2 : 3 was observed (Buck and Keister, unpublished data).

The present evidence of the subordinate physiological position of the anterior spiracles agrees also with opinions of Strasburger (1935) on *Drosophila* and Levenbook (1951) on *Gastrophilus.*

None of the evidence on the relative "capacities" of the two pairs of spiracles bears on the question of whether gas transport is via diffusion or ventilation. This problem will be dealt with elsewhere.

Summary

1. In the *Phormia regina* larva oxygen uptake from air at 25° of larvae with both pairs of spiracles ligated off is about 0.09 μL./mg. live wt./hr. (10% of normal respiration) and increases four-fold in oxygen. This is the maximum cutaneous uptake possible from air.

2. The posterior pair of spiracles by itself can admit enough oxygen from air to supply normal needs.

3. On the basis of reciprocal ligation experiments, the anterior spiracles have about half the "capacity" of the posterior spiracles for admitting oxygen into the tracheal system, and can, by themselves, admit about 60% of normal needs.

4. The internal pO_2 in larvae with both pairs of spiracles ligated is zero, even in an atmosphere of pure oxygen.

5. In normal (non-hypoxic) larvae, cutaneous respiration accounts for less than 2.5% of the total oxygen uptake.

6. The permeability of the integument to oxygen is of the order of 0.005 μL./mm.²/min./atm.

7. DDT does not affect cutaneous permeability to oxygen.

8. DDT-poisoned larvae cannot obtain enough oxygen from air even with all spiracles and skin functional, the hypoxia increasing nonlinearly in the order unligated < anteriorly ligated < posteriorly ligated < doubly ligated. Even in pure oxygen the larvae are hypoxic if either pair of spiracles is cut off.

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THE CORRELATION OF DESOXYRIBONUCLEIC ACID SYNTHESIS AND THE RATE OF RESPIRATION IN THE SEA URCHIN EMBRYO[1]

JEAN J. COMITA AND ARTHUR H. WHITELEY

Department of Zoology and the Friday Harbor Laboratories, University of Washington, Seattle 5, Washington

Measurements of the rate of respiration of sea urchin eggs from fertilization through early development have been reported by Warburg (1915), Gray (1925, 1926), Rapkine (1927), Ephrussi (1933), Tyler (1936b), Lindahl (1939), Horowitz (1940), Hutchens *et al.* (1942), Borei (1948), and others. In all experiments the rate of oxygen uptake is shown to increase during development. A number of attempts have been made in the past to learn about factors controlling, or related to, this increase. It seems not to be related closely to the increase in total nuclear volume (Godlewski, 1908), the increase in the number of cells of the embryo (Warburg, 1915; Tyler, 1936a), nor to the increase in the rate of cleavage of the cells of the embryo (Gray, 1926; Tyler, 1936b).

An experiment that suggests a possible relation between the rate of respiration and the content of desoxyribonucleic acid (DNA) was done by Brachet (1938). The oxygen consumption of fertilized eggs of *Chaetopterus* as well as eggs activated by KCl was measured and the respiration of the KCl-activated eggs, which differentiate without cleavage, was found to increase at a much slower rate than that of the fertilized eggs. It was found that the larvae from the KCl-activated eggs contained only 30% of the DNA present in the fertilized eggs several hours after the activation or fertilization. Brachet's interpretation was that (p. 97) "both the reduced oxygen uptake and the slower development are linked together; such a conclusion could support Tyler's opinion that part of the energy available in the egg is needed for the growth and differentiation processes taking place during development." Similarly, Tyler and Horowitz (1938) concluded, from an analysis of the effects of phenylurethane on respiratory rates of *Urechis* embryos, that the normal rise in rate of respiration is probably connected with nuclear division.

The purpose of the work recorded here has been to determine whether a correlation does exist between the rate of oxygen consumption and the DNA content of sea urchin embryos. If such a relationship were found, another conclusion, alternative to Brachet's, could be drawn: it would be possible that DNA, as a constituent of the genome of the cell, is limiting the production or activation of respiratory enzymes which would be responsible for the rate of oxygen consumption.

This problem was undertaken at the suggestion of Dr. Albert Tyler, who had calculated from published figures that a close relation between these two events was

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likely. The authors are indebted to Dr. Tyler for proposing the investigation and for critically reading the manuscript.

Materials and Methods

The eggs of *Strongylocentrotus purpuratus* (Stimpson), collected at San Juan Island, Washington, were used in this study. For each experiment the eggs of only one sea urchin were used. To induce spawning of both sexes the urchins were injected with 4.25% KCl (Tyler, 1949). The eggs were washed with filtered sea water at least three times or until 95 to 100% fertilization of the eggs was attained. If near 100% fertilization were not possible, or if the eggs were abnormal in any respect, they were not used.

From a 5% suspension of eggs, 5- or 10-ml. aliquots were taken for egg counts and for the determination of oxygen consumption and DNA content of the unfertilized eggs. After dilution of the remaining egg suspension the eggs were fertilized, washed free of excess sperm, and the original egg concentration re-established. Aliquots of approximately 10 ml. were then taken for determinations at the two-cell stage; for all later stages 10-ml. aliquots were added to 200 ml. of filtered sea water for the first experiment and to 500 ml. for the second experiment. The embryos were cultured in a constant temperature bath at 18° C. If development did not proceed normally with the survival of more than 85% of the embryos, the results were not used.

For the determination of the rate of oxygen consumption and quantity of DNA, the embryos were harvested by centrifuging. For the swimming stages the centrifuging was done with a modified Foerst plankton centrifuge constructed so that the embryos were in contact with glass or Plexiglas only. The embryos were subjected to a maximal RCF of 150 × g for 15 to 20 minutes. They showed no visible evidence of injury following this treatment.

The number of eggs was used as a parameter for the measurement of the DNA content and the rate of oxygen consumption. Egg counts were made on an aliquot of the unfertilized eggs which was diluted until there were from 100 to 200 eggs per 10 ml. Five to 10 counts were made of 10-ml. aliquots of this dilution. The aliquots were transferred to a narrow, shallow plastic trough where the eggs settled in a row and could be counted readily at low magnification. The variation in the egg counts was less than 10%.[2] This need only be considered when comparing the first experiment with the second where different egg batches were used.

As soon as possible after harvesting, the respiration of the embryos was measured with the Warburg constant volume respirometer. The concentration of eggs used varied from 5 to 10% depending on the stage of development of the embryos. The speed of shaking used was 120 cycles per minute with an amplitude of 2.5 centimeters. These conditions of crowding and shaking were not harmful to the embryos, as determined by examination after respiration measurements. The temperature at which the measurements were made was 18 ± 0.01° C.

After the rate of respiration had been determined over a period of from ½ to 2

[2] In Experiment 1, the average number of eggs is 56,000 per ml., and the 95% confidence interval is 55,300 to 56,600. In Experiment 2, the average number of eggs is 51,800 per milliliter with a 95% confidence interval of 49,400 to 54,100.

hours, the embryos were removed quantitatively from the Warburg flasks, centrifuged mildly, and the excess sea water removed. The packed embryos were then frozen at − 35° C. until they were analyzed for DNA content. The period of frozen storage was about two weeks.

The analyses for DNA were based on determining the amount of DNA phosphorus and were made by a combination of parts of the methods of Schmidt and Thannhauser (1945) and Schneider (1945) with the modification for the direct analysis of DNA by Schmidt, Hecht and Thannhauser (1948). The separated DNA was analyzed for total phosphorus content by the method of Berenblum and Chain (1938) following perchloric acid digestion.

Abrams (1951) reports that re-precipitation of the DNA is necessary for the removal of all of the contaminating RNA. To determine if this were necessary for the material used in the present investigation, DNA determinations were made on two aliquots of unfertilized eggs without re-precipitation of the DNA and on two aliquots with re-precipitation of the DNA two times. This is a severe test of the

TABLE I

The effect of re-precipitation of DNA on its quantitative determination

Unfertilized egg samples	Micrograms DNA P/10^6 eggs	Average
Washed twice	0.932 0.988	0.960
Precipitated twice	0.859 0.942	0.901

method because the ratio of RNA to DNA is very high in the unfertilized egg. The results are given in Table I.

The difference in DNA phosphorus obtained by the two methods may be considered negligible. Nevertheless, in the second experiment all of the samples of DNA were re-precipitated once and washed once.

RESULTS

To determine whether a correlation exists between the rate of respiration and the content of DNA phosphorus in the normal development of the sea urchin embryo, these were measured at intervals between fertilization and the early pluteus stage. The first series, Experiment 1, was carried out in February, 1951, and the second, Experiment 2, in August, 1951. Each series consists essentially of two parallel experiments in that both the respiration and DNA phosphorus determinations were made on duplicate aliquots of eggs from the original batch with the exception of the first three DNA phosphorus determinations of Experiment 1. The results are shown in Figure 1.

The typical increase in the rate of respiration is indicated by the upper two curves. Quantitatively the uppermost of these is very similar to that published by Horowitz (1940) for this species. The curves for the amount of DNA phosphorus in the embryos parallel those of the rate of respiration except for the initial hour or two during which the latter increase rapidly and the former do not. The final

plateau of the curves of Experiment 1 may be the result of starvation; the larvae do not develop further than the short-armed pluteus stage without feeding.

These data indicate that in both experiments the respiration curves parallel the corresponding curves for DNA phosphorus. To help determine whether any relationship exists between the two phenomena, correlation coefficients were calculated. The coefficient of correlation, r, for the combined data of both experiments, covering the developmental period from the 2-cell stage to the pluteus, with fourteen paired values of rates of oxygen consumption and amounts of DNA phosphorus, is 0.936. This excludes the values for the unfertilized egg, which is assumed to be very different physiologically from the developing egg. The correlation coefficient

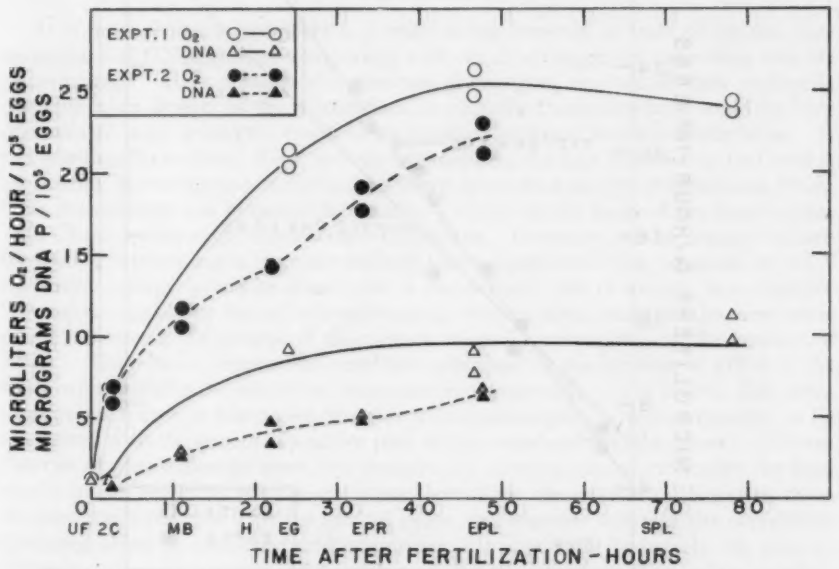

FIGURE 1. Rate of oxygen consumption and DNA phosphorus content in the embryos of *Strongylocentrotus purpuratus*. Developmental stages: UF, unfertilized; 2C, two-cell; MB, middle blastula; H, hatching; EG, early gastrula; EPR, early prism; EPL, early pluteus; SPL, short-armed pluteus.

for the second experiment alone, for which the data are more complete with ten paired values, is 0.978. These correlation coefficients are much higher than necessary to be significantly different from zero at the 1% level. A scatter diagram with the regression lines for the two experiments is shown in Figure 2.

In the unfertilized egg the rate of oxygen consumption, from the data of the first experiment, is 1.24 microliters per hour per egg while the rate of 8.34 microliters per hour per egg is the value predicted from the regression line $y = 2.04x + 6.05$ knowing that the amount of DNA phosphorus is 1.12 micrograms in the unfertilized egg. The observed value is not quite significantly different from the calculated one at the

5% level.[3] Unfortunately the amount of DNA phosphorus and the rate of oxygen consumption previous to fertilization for the eggs used in Experiment 2, for which the standard error around the regression line is much less than that for the combined experiments, were not determined so that the test for significance cannot be employed using only this second experiment. However, the data for respiration and DNA phosphorus in the unfertilized egg have been confirmed many times in other experiments by ourselves and other workers using the methods employed here, and it is quite likely that a significant difference between the respiration in the unfertilized egg and the amount expected from the regression line would be found. It may be

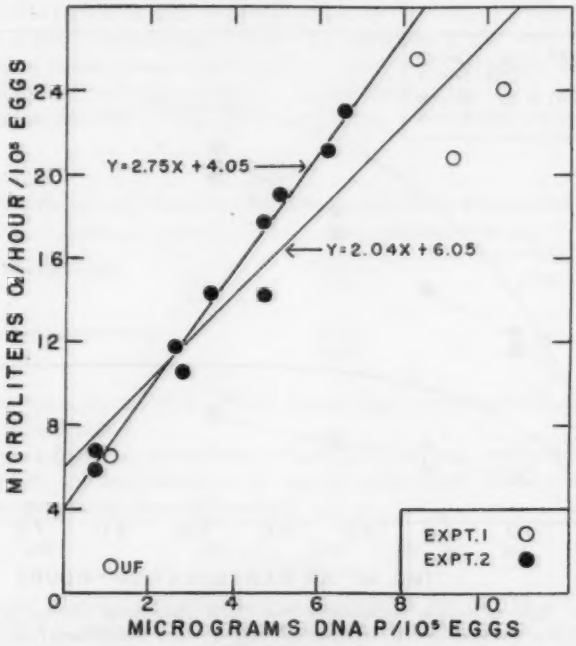

FIGURE 2. Scatter diagram with regression lines for the rate of oxygen consumption, y, and amount of DNA phosphorus, x, in developing embryos. The equation of the line for Experiment 2 is $y = 2.75x + 4.05$ and for the combined experiments is $y = 2.04x + 6.05$.

tentatively concluded that in the unfertilized egg respiration is not related to DNA in the same manner as it is in the developing embryo.

The curves obtained in Experiment 1 (Fig. 1), both for the rate of respiration and DNA synthesis, are higher than those of Experiment 2. Experiment 1 was done in February during the height of the breeding season whereas Experiment 2

[3] The t test was used for this determination. A value of 2.18 for t is necessary for significance at the 5% level for 12 degrees of freedom, and the calculated value was -2.12; therefore, the probability is approximately 0.06.

was done in August, so that the dissimilarity could be due to a difference in winter and summer eggs. It was found by Fox (1938) that at a given temperature of about 20° C. for *Psammechinus* and *Paracentrotus,* material from colder waters cleaved more rapidly than material from warmer waters. In an analogous way, it may be that the rate of oxygen consumption and the synthesis of DNA would be greater for winter eggs than summer eggs when measured at the same temperature. Also, in each experiment all of the analyses were made on the eggs of one sea urchin so that the difference in the curves may be due to individual variation. The differences obtained cannot be accounted for by the variation in the egg counts.

Discussion

It is most probable that there is a relationship between the rate of oxygen consumption and DNA synthesis beginning with the 2-cell stage and extending into the pluteus stage. It is possible that the two phenomena are related only indirectly through other aspects of the metabolism; such indirect relationships could be very complicated and would be fruitless to consider without further information. If the relationship is direct, there are two possibilities, the first of which is that such a correlation exists because of the use of oxygen to produce energy to synthesize DNA. This relationship was indicated by Brachet (1938) on the basis of his experiments with *Chaetopterus* eggs, which were cited earlier. However, aerobic energy is used for many purposes and it is rather unlikely that a limitation of the synthesis of DNA by aerobic energy would be observable in the over-all rate of oxygen consumption. The second possibility for a direct relationship is more likely; it is that the correlation exists because of the control of the rate of oxygen consumption by the amount of DNA. The rate of respiration could be controlled by the amount of DNA if the latter were limiting the synthesis or activation of enzymes. It is known that chromosomes are largely composed of desoxyribonucleoprotein which is thought to be associated with the genetically active part of the chromosomes, the genes. Current theories of gene action propose that the primary effect of genes is to confer the final specificity on enzymes, usually one gene controlling one enzyme. However, these theories are concerned with the kind of genes and enzymes whereas the correlation discussed above is concerned with quantities. It would not be merely the number of genes present at any stage of development that dictates the activity of the respiratory enzymes, because, as was pointed out earlier, the number of cells, and, therefore, the number of genes, bears no relation to the respiration. It must be concluded, if a cause and effect relation is found to exist here, that the amount of respiratory activity is dependent on the total amount of DNA present in the embryo.

It was tentatively concluded that the respiration of the unfertilized egg is lower than expected judging from the amount of DNA phosphorus present. It is possible that before fertilization the activity of enzymes previously stored in the eggs is suppressed and respiration is low. After fertilization these enzyme systems could function at a greater capacity possibly due to a protoplasmic rearrangement (Brachet, 1950). This rearrangement is reflected in the increased rate of oxygen consumption immediately following fertilization, which, therefore, does not need to involve synthesis of DNA or new enzymes. After the utilization to full capacity of these stored enzymes, the synthesis or activation of more enzymes would be dependent on the

synthesis of DNA, and from then on, the rising rate of oxygen consumption would parallel the increasing amount of DNA.

The values obtained for DNA phosphorus in this investigation approximate those obtained by Schmidt, Hecht and Thannhauser (1948) and Villee, Lowens, Gordon, Leonard and Rich (1949). Both groups of workers analyzed the eggs of *Arbacia punctulata* by the method of Schmidt and Thannhauser. Schmidt and coworkers found that the DNA phosphorus per embryo varied between 0.6 and 0.9×10^{-5} micrograms in the unfertilized egg and increased to 10.7×10^{-5} micrograms in the 24-hour pluteus. Villee and co-workers found an increase from 1.2×10^{-5} to 7.0×10^{-5} micrograms of DNA phosphorus per embryo from the third to the twentieth hour of development. The comparable results of the present study are 1.1×10^{-5} micrograms of DNA phosphorus per embryo in the unfertilized egg and 10.4×10^{-5} micrograms in the pluteus.

Summary

1. Following an abrupt rise in rate of respiration of the egg of the sea urchin at the time of activation there is a period of continued increase extending into the pluteus larval stage. This is not explainable by means of the increase in cell number, cleavage rate, or nuclear volume.

2. In the developing embryo of *Strongylocentrotus purpuratus* (Stimpson) the increase in the rate of oxygen consumption has been found to have a very high positive correlation with the amount of desoxyribonucleic acid (DNA) phosphorus present in the embryo. The correlation coefficient was found to be much higher than necessary to be significantly different from zero at the 1% level. The regression coefficient for the linear regression of the rate of respiration on the amount of DNA phosphorus for the best studied case was found to be 2.75 microliters of oxygen per microgram of DNA phosphorus per hour.

3. The same correlation probably does not exist in the unfertilized egg.

4. If the relation is a direct one, the most reasonable interpretation of the correlation found is that the synthesis or activation of respiratory enzymes is under nucleic acid control.

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MOULTING AND GROWTH IN BALANUS IMPROVISUS[1]

JOHN D. COSTLOW, JR. AND C. G. BOOKHOUT

Duke University Marine Laboratory, Beaufort, N. C. and Department of Zoology, Duke University, Durham, N. C.

The Cirripedia differ from other arthropods in that the body is enclosed by calcareous plates which are not shed with the chitinous layers of the animal body and the inner lining of the mantle. As early as 1854 Darwin reported that members of the families Balanidae and Chthamalidae underwent periods of exuviation. He reports that Mr. W. Thompson of Belfast kept twenty specimens of *Balanus balanoides* alive, presumably in the laboratory, and on the twelfth day all had moulted once and one individual twice. The frequency of moulting over an extended period of time has not been determined accurately for any sessile barnacle, presumably because of the difficulties involved in maintaining them in the laboratory. Thomas (1944) reports that moults of a selected group of *Balanus perforatus* of uniform size but unknown age occurred about every fifth day but makes no reference to the period over which these observations were made. Other than these observations there has been no statement, to our knowledge, concerning the relationship between growth of shell plates and ecdyses nor is the frequency of moulting and the intermoult period, from the time of settling, known for any length of time in any barnacle.

The purpose of this investigation was to determine, therefore, a medium which would support growth of barnacles in the laboratory, to ascertain the time of moulting, period of intermoult, and the relationship of shell growth to the moulting cycle. *Balanus improvisus* Darwin, a barnacle which settles at Beaufort, N. C. during the winter and spring months, was selected for this study.

Methods

Young *B. improvisus* were collected by suspending plastic squares 6″ × 6″ in the Beaufort Harbor for 24 hours. When brought into the laboratory most of the barnacles were in the pinhead stage but some were still cyprids. The latter were not considered a day old until the following day when metamorphosis was complete. Small pieces of plastic containing one or two pinhead barnacles were cut from the larger squares and placed in watch glasses which were in turn set, but not immersed, in finger bowls of sea water. Culture media of *Nitzschia closterium*, *Chlorella* sp., and *Chlamydomonas* sp. were obtained from Dr. T. Rice of the Shellfish Laboratory, U. S. Fish and Wildlife Service, Beaufort, N. C. and placed in watch glasses. After initial cultures were obtained from Dr. Rice, we maintained our own and sub-cultured regularly.

Daily examinations of the barnacles and surrounding medium were made to determine whether a moult was present. At this time the old culture medium was replaced by fresh.

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Four separate series of cultures of *B. improvisus* were maintained. The first series was started on March 30, 1952 with 56 one day-old barnacles and followed through 64 days. A second series was begun with 100 one day-old "pinheads" and maintained for 57 days. This series was supplied with a more uniform food source and was used for fixed material for another study. A third series of 10 barnacles was started and cultured in autoclaved, filtered sea water with no food source and maintained for 13 days. These were used to make a critical analysis of the time of the first moult. The fourth series was initiated with 38 barnacles and used principally as a source of fixed material for the first five moults. On day 32 of the first series and day 22 of the second all barnacles were transferred to plastic compartmented boxes to facilitate feeding and observation. These were placed under daylight fluorescent lamps and maintained under the same conditions as the cultures of algae. The average temperature of the culture media in these studies was 20° C.

Daily rostral-carinal and lateral measurements of 204 barnacles in the four series were made and areal increments were computed to the nearest 0.01 sq. mm. Each barnacle was measured with an ocular micrometer mounted in a binocular dissecting microscope. In addition, two series of 20 and 30 barnacles, collected on glass plates and suspended in the Beaufort Harbor, were measured daily for 26 and 12 days, respectively, to obtain information concerning growth under natural conditions as compared with those grown in laboratory cultures.

Results and Discussion

Moulting

It has been determined from this study that *B. improvisus* undergoes frequent and regular ecdyses. The results are given in Tables I, II, III, and IV and Figures 1, 2, and 3.

Series I, consisting of 56 barnacles, passed through 20 moults in the course of 63 days. This series received only that food which was available in sea water from day 1 to 6. On day 6 they were placed in an autoclaved and filtered culture of *Nitzschia closterium*. This was changed daily for 25 days, at which time a mixed culture of *Chlamydomonas* sp. and *Nitzschia closterium* was used. An examination of Figure 1 and Table I indicates that *B. improvisus* will complete the first few moults even though an adequate food source is not available. This would seem to imply that the animal possesses stored food which is carried over from the cyprid to the pinhead, as reported by Doochin (1951). Thereafter an adequate source of food must be available or the barnacles will die. The first nine moults are confined to a more limited span than those from moult 10–20 (Fig. 1). However, if each individual on the curve is considered there is uniformity in the intermoult period. The average intermoult period for 40 barnacles of this series is shown in Table II. The variation in time exhibited by certain individual barnacles must be due to something other than food source since this was the same for each barnacle, even though cultured separately.

The second series of 100 barnacles was fed a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. from day 1 through day 57. After 57 days most of this series was fixed, but a few individuals were maintained on *Chlamydomonas* sp. alone from day 57 to day 120, and records kept of the moulting frequency. Examination

of Table III and Figure 2 shows that the moulting frequency is more uniform from the beginning and does not exhibit a pronounced lag period until after the 13th moult. Also, the intermoult periods remained relatively uniform even through the 42nd moult, covering a period of 120 days. Otherwise, the results are similar to those of the first series.

Prior to the determination of frequency of ecdysis the second moult had been considered the first. After it was determined that moulting occurred on the average of every 2–3 days, the possibility of an earlier moult was suggested which might have been overlooked. Therefore, a third series of 10 barnacles was cultured on filtered, autoclaved sea water with no food source, to make a critical analysis of the time of the first moult. Since it had been determined earlier that food was not necessary for the first few periods of ecdyses it was omitted so that a moult, if present, could be detected more readily. To insure as much uniformity of age as possible newly attached cyprids were collected and the time of metamorphosis was noted. Two

TABLE I

Moulting periods and percentage of moults in Balanus improvisus
*Series I**

Day	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Moult 2	4	24	9	8	4	1									
	8	48	18	16	8	2									
3			1	5	16	11	8	7	2						
			2	10	32	22	16	14	4						
4						2	7	20	9	8	3	1			
						4	14	40	18	16	6	2			
5									7	19	13	7	1	2	1
									14	38	26	14	2	4	2

Day	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Moult 6	2	6	16	11	6	5	3	1							
	4	12	32	22	12	10	6	2							
7			2	2	14	12	9	6	3						
			4.17	4.17	29.4	4.25	18.7	12.5	6.26						
8					1	3	7	15	9	7		2	1		
					2.23	6.68	15.5	34.4	20.0	15.5		4.45	2.23		
9								3	7	19	9	6	2	1	2
								6.8	15.9	34.1	20.4	13.6	4.55	2.28	4.5

Day	23	24	25	26	27	28	29	30	31	32	33	34
Moult 10	3	3	10	11	5	5		1	2			
	7.5	7.5	25	27.5	12.4	12.4		2.5	5			
11			3	1	8	12	6	3	1	4	1	1
			7.5	2.5	20	30	15	7.5	2.5	10	2.5	2.5

First line represents the number of moults for each day.
Second line represents the per cent of moults for each day.
* Day 1–6, sea water only; day 6–33, *Nitzschia;* day 34–46, *Nitzschia-Chlamydomonas.*

TABLE I—*Continued*

Day	27	28	29	30	31	32	33	34	35	36	37	38	39
Moult 12	3 7.5		3 7.5	9 22.5	8 20	6 15	4 10	2 5	4 10	1 2.5			
13			1 2.5	2 5	1 2.5	3 7.5	7 17.4	12 30	6 15	4 10	4 10		
14					1 2.5		1 2.5	6 14	9 22	9 22	9 22	3 7.5	3 7.5
Day	33	34	35	36	37	38	39	40	41	42	43	44	45
Moult 15	1 2.5		2 5	6 15	6 15	12 30	7 15.5	4 10	1 2.5	1 2.5			
16			1 2.5		4 10	3 7.5	7 15.5	9 22.5	8 20	5 12.5	2 5	1 2.5	
Day	38	39	40	41	42	43	44	45	46	47	48	49	50
Moult 17	1 2.5	2 5	3 7.5	7 17.5	7 17.5	9 22.5	5 12.5	1 2.5	1 2.5	1 2.5	2 5	1 2.5	
Day	40	41	42	43	44	45	46	47	48	49	50	51	52
Moult 18	1 2.5	1 2.5	1 2.5	7 17.5	1 2.5	2 5	6 15	5 12.5	14 22.5	3 7.5	1 2.5	1 2.5	2 5
Day	43	44	45	46	47	48	49	50	51	52	53	54	55
Moult 19	1 2.5	2 5			4 10	6 15	7 17.5	4 10	6 15	4 10	3 7.5	1 2.5	2 5
20				1 2.5			2 5	6 15	4 10	11 27.5	4 10	1 2.5	2 5
Day	56	57	58	59	60	61	62	63	64				
Moult 20	3 7.5	3 7.5	2 5				1 2.5						

TABLE II

Average intermoult periods of 40 Balanus improvisus
Series I

Intermoults	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–12
Days	3.23	2.6	2.15	2.3	2.25	2.23	2.18	2.4	2.48	2.78

Intermoults	12–13	13–14	14–15	15–16	16–17	17–18	18–19	19–20	20–21
Days	2.58	1.95	1.98	2.13	2.73	3.63	3.35	3.18	3.62

days after metamorphosis 9 out of the 10 barnacles passed through the first moult, whereas the tenth died in the process of moulting. The moults were observed through a compound microscope (110 ×) so that proper identification could be made. It is now believed that barnacles of all the other series moulted on the second day, but because of the small size of the exuviae and the masking effect of the particulate food matter, they were not detected.

The fourth series of 38 barnacles was maintained on *Chlamydomonas* sp. from day 1 and used principally as a source of histological material for another study. The moulting periods and percentage of moults of this series are shown in Table IV

TABLE III

*Moulting periods and percentage of moults in Balanus improvisus Series II**

Day	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Moult 2	1 1	44 45	44 45	7 7			1 1							
3				10 10	35 36	48 49	5 5							
4						3 3	24 25	48 49	19 20	1 1	1 1			
5								4 4	26 27	42 43	21 22	3 3		
6										4 4	30 31	39 40	21 22	1 1
Day	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	
Moult 7	6 7	29 32	35 39	17 19	2 2									
8			5 6	29 34	25 29	15 17	9 10		2 2					
9					5 6	21 24	26 31	16 18	11 13	5 6	1 1			
10							5 6	14 17	35 43	19 23	6 8	2 2		
Day	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	
Moult 11	11 14	19 24	35 45	7 9	3 4	1 1	2 2							
12		2 3	15 19	30 38	19 24	6 8	2 3	2 3	1 1					
13				5 7	15 21	28 39	16 22	1 1	3 4	2 3		2 3		

First line represents the number of moults each day.
Second line represents the per cent of moults for each day.
* Fed a mixed culture of *Nitzschia* and *Chlamydomonas* from day 1–57.

TABLE III—*Continued*

Day	27	28	29	30	31	32	33	34	35	36	37	40			
Moult	1	6	8	23	18	5	1	1	1	1	1	1			
14	1.5	9	12	34	27	7	1.5	1.5	1.5	1.5	1.5	1.5			
Day	28	29	30	31	32	33	34	35	36	37	38	40	43		
Moult	1	2	4	6	19	11	8	4	1	3	3	2	1		
15	1.5	3	6	9	29	17	12	6	1.5	4.6	4.6	3	1.5		
Day	30	32	33	34	35	36	37	38	39	40	41	42	44	45	
Moult	2	6	2	5	6	5	15	10	3	3	1	5	1	1	
16	3	9	3	8	9	8	23	15	4.6	4.6	1.5	8	1.5	1.5	
Day	32	33	34	35	36	37	38	39	40	41	42	43	44		
Moult	2		3	2	2	2	7	7	11	8	7	5	3		
17	3		4.6	3	3	3	11	11	17	12	11	8	4.6		
				1		2	2	3	2	10	9	11	3		
18				1.5		3	3	4.6	3	17	15	20	4.6		
								2	1	3	7	7	5		
19								4	2	6	14	14	10		
										1	2	4	1		
20										3	5	11	3		
													3		
21													13		
Day	45	46	47	48	49	50	51	52	53	54	55	56	57		
Moult	2	1	1	1											
17	3	2	2	2											
	6	5	2	2			1								
18	10	8	3	3			1.5								
	5	4	7	2	2		1		2	1	1				
19	10	8	14	4	4		2		4	2	2				
	7	9	2	1	1	1	2	1	1		1	2	1		
20	18	24	5	3	3	3	5	3	3		3	5	3		
	4		4	4	1		1	1	1	2	1	1			
21	17		17	17	4		4	4	4	8	4	4			

and Figure 3. The sequence of moulting and the uniformity of intermoult periods appear to be related to the availability of food. From an examination of Figures 1, 2, and 3 it can be seen that barnacles maintained on a diet of *Chlamydomonas* sp. showed more uniformity than those maintained on a mixed diet of *Nitzschia closterium* and *Chlamydomonas* sp. It is our belief that this is associated with the motility of *Chlamydomonas* sp., making it more readily available to the barnacles, whereas the diatom *Nitzschia closterium* tended to clump and settle to the bottom becoming less readily available even though the culture medium was changed daily.

Kruger (1940) reported that in the stalked barnacles a new ring is added to the terga and scuta at each moult since the opercular membrane is shed with the exuvae of the body proper. An exception to this is found in members of the family Coronulinae in which the old opercular membrane is retained. The possibility of addition of new rings to the opercular plates at the time of moulting in *B. improvisus* was, therefore, investigated. We can concur that the chitinous ring is shed but have found no evidence that there is any correlation between ring number and the number of moults. In *B. improvisus,* reared from day of settling through the 42nd moult, the number of rings never corresponded to the number of moults.

TABLE IV

*Moulting periods and percentage of moults in Balanus improvisus Series IV**

Day	2	3	4	5	6	7	8	9	10	11	12	13
Moult 1	4											
			25	8								
2			78	22								
				4	7	15	2					
3				14	25	54	7					
					1	5	11	3	1	2		
4					4	22	48	13	4	8		
							1	2	6	2	1	
5							7	14	43	14	7	
									1	1	3	1
6									16	16	50	16

First line represents the number of moults each day.
Second line represents the per cent of moults for each day.
* Fed on *Chlamydomonas* from day 1–15.

Growth

Numerous studies of the growth of various species of barnacles have been made in different parts of the world. However, the literature contains no reference to growth rates of barnacles raised on uni-algal cultures in the laboratory which will now be considered.

From the daily measurements of rostral-carinal and lateral diameters the daily increase in area of basis was computed. From an examination of Figure 5 one may conclude that barnacles fed on *Chlamydomonas* sp. from day 1 immediately show a more rapid growth than those fed on a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. This is true even though the average area of the basis of the barnacles of Series IV was smaller on day 1. It is not surprising to note that barnacles fed grew more rapidly from day 1–6 than those without nourishment other than that which was present in standing sea water. It may be noted that from day

1–13 growth is slow, from 13 to 22 days there is an increase, but from 22 days to 37 and 46 days, respectively, the accumulated increase is even greater. It appears as though growth is following an S curve.

A comparison of the average growth of 10 barnacles grown in the Beaufort Harbor with 10 grown in the laboratory on *Chlamydomonas* sp. is shown in Figure 5. The 10 in the Harbor were eliminated at twelve days by sheepshead at the end of the settling period of *B. improvisus*. In the spring of 1953, therefore, the study

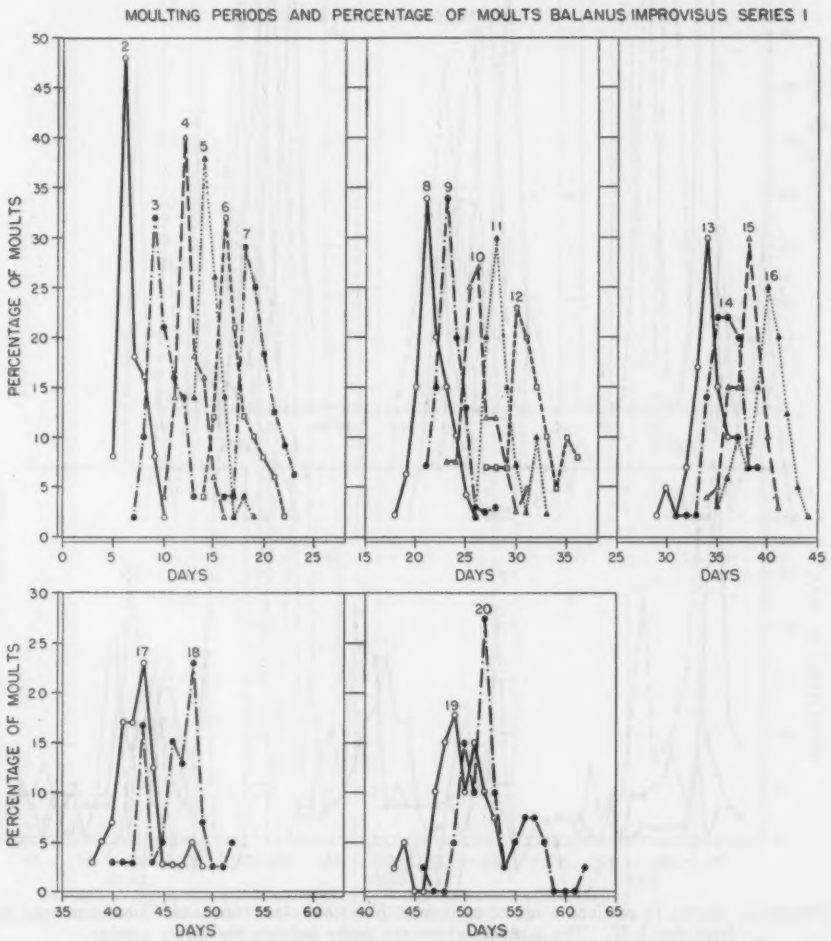

FIGURE 1. Series I received no supplemental food from day 1–6, from day 6–25 was fed *Nitzschia closterium*, from day 25–63 received a mixed culture of *Chlamydomonas* sp. and *Nitzschia closterium*. The numbers above the peaks indicate the moult number.

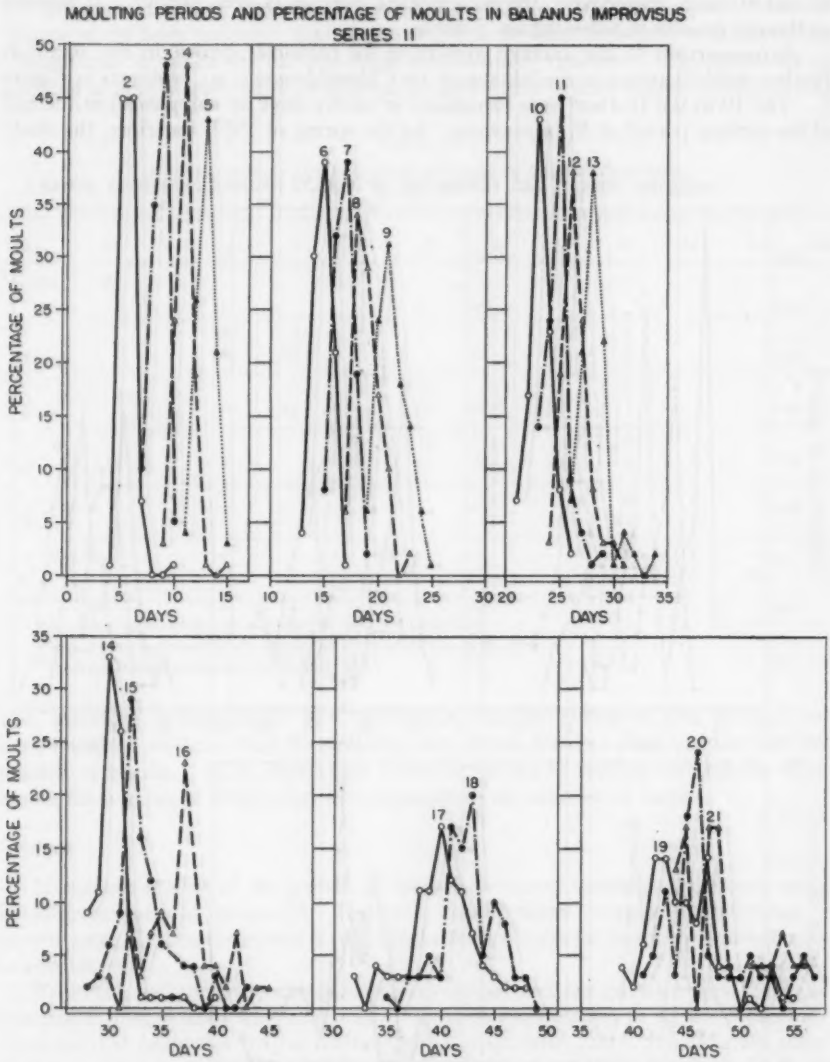

FIGURE 2. Series II received a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp. from day 1–57. The numbers above the peaks indicate the moult number.

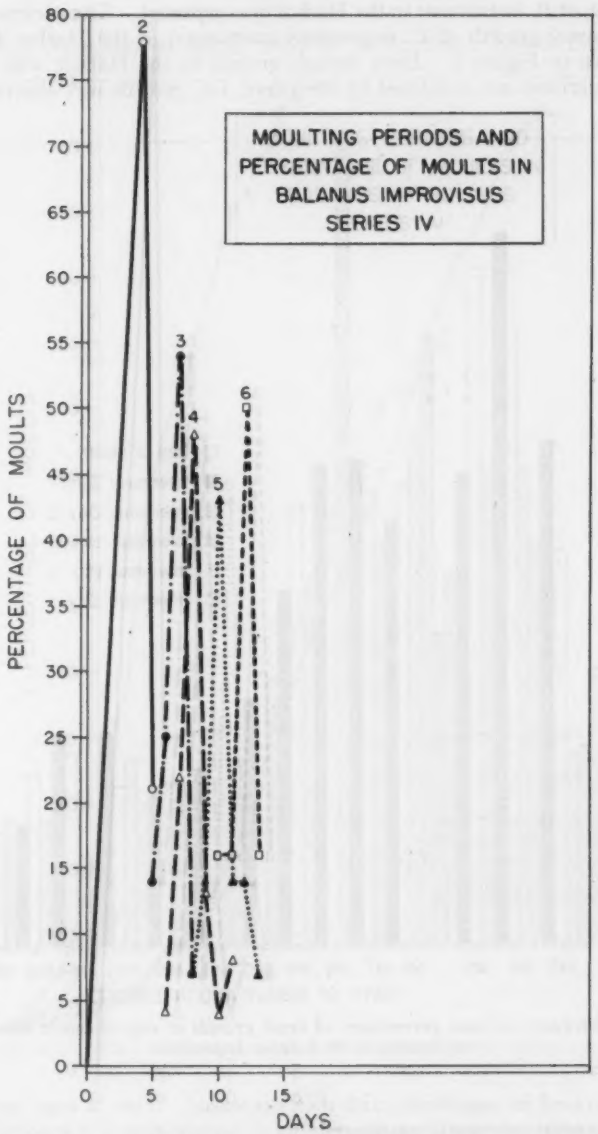

FIGURE 3. Series IV received *Chlamydomonas* sp. from day 1–13. The numbers above the peaks indicate the moult number.

of the growth of *B. improvisus* in the Harbor was repeated. The accumulative daily increase of areal growth of *B. improvisus* maintained in the Harbor for 26 days is also shown in Figure 5. Even though growth in the Harbor was greater the same characteristics are exhibited by the curve, *i.e.*, growth was slower in the be-

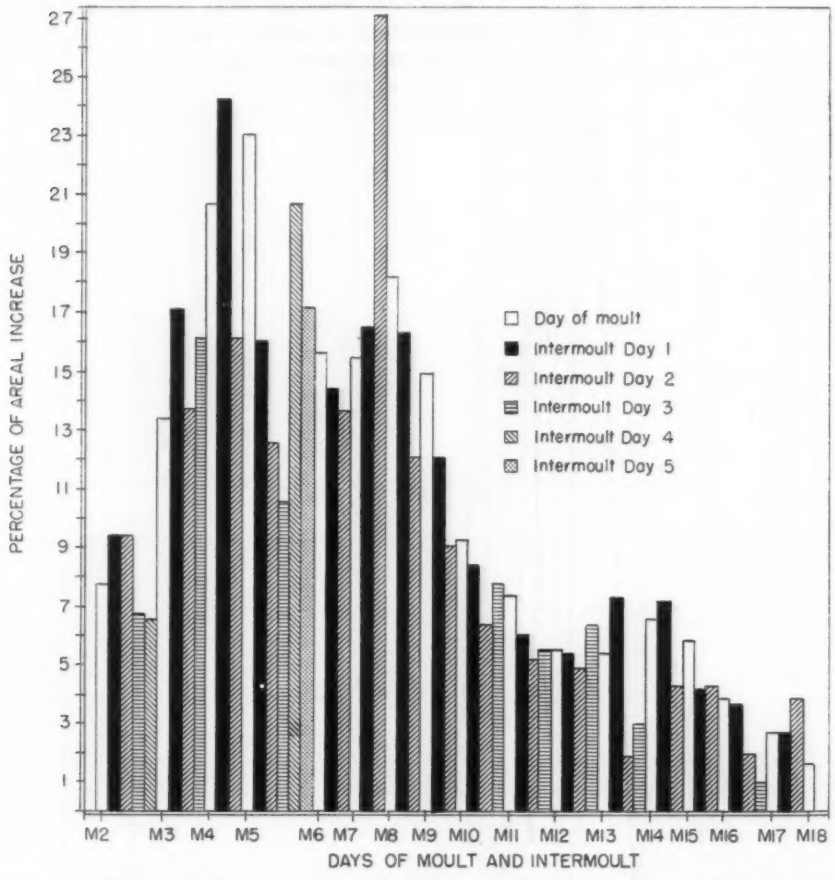

FIGURE 4. Histogram to show percentages of areal growth at day of moult and each day of intermoult in 39 *Balanus improvisus*.

ginning, increased in magnitude, and then decreased. This is even more evident if the per cent areal increase is considered.

An analysis of Figure 5 reveals that while the uni-algal cultures of *Nitzschia closterium* and *Chlamydomonas* sp. did not maintain a growth rate equal to that found under natural conditions where the food supply was more varied and pre-

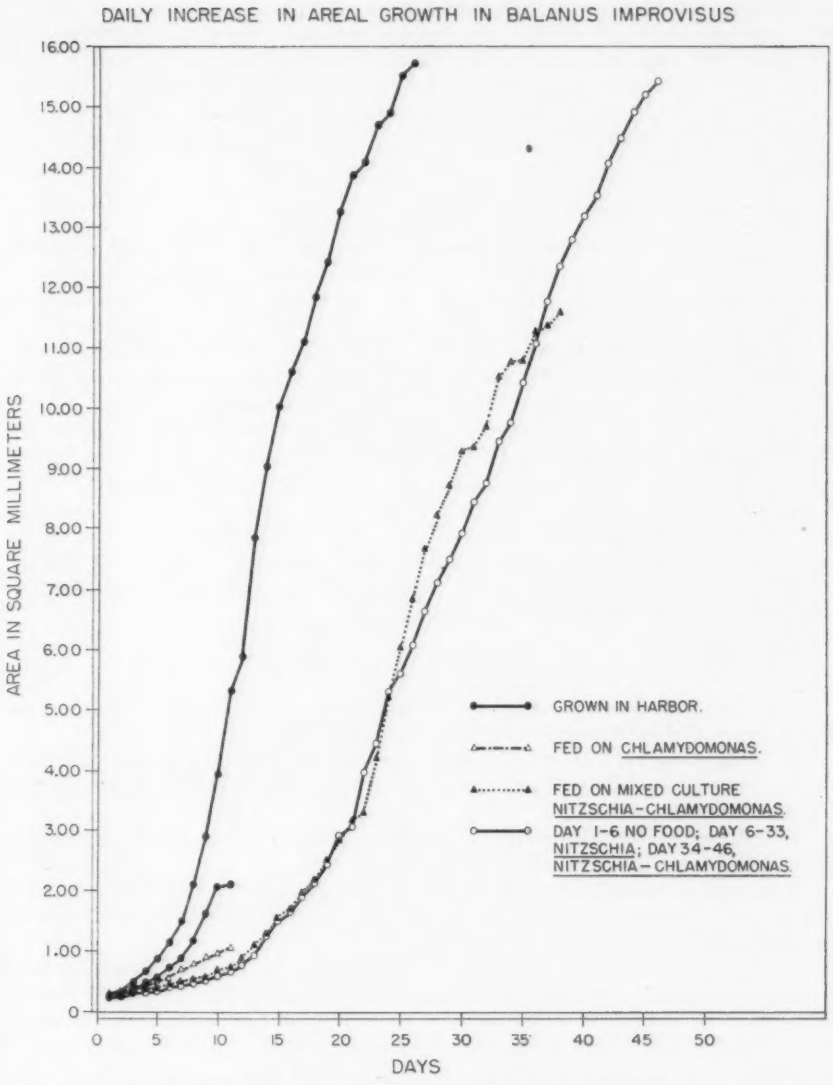

FIGURE 5. Comparison of daily increase in areal growth in *Balanus improvisus* grown in the Harbor and reared in the laboratory.

sumably of a greater magnitude, the resulting rate under artificial conditions was continuous and the food source considered adequate since the mortality under these conditions amounted to only 0.5%. The factors of social competition, crowding and predation, which appear to account for the high mortality observed in nature, were eliminated by the complete segregation of the individual barnacles in the laboratory. It is believed that the absence of water currents in the laboratory, which in the natural environment are the chief conveyers of food, would account to some extent for the lower growth rate.

Shell growth and moulting

There is no indication of any correlation between shell growth and moulting. Growth at the day of certain moults is less than days of intermoult but at other times, in the same barnacle, there is more growth at the day of moult than before or afterwards. From this type of analysis it must be concluded that growth in *B. improvisus*, although continuous, is erratic. Since individual growth rates did not show a definite trend, it was thought that an average of a group of barnacles might. The results are given in Figure 4. There is a general increase in the per cent of areal growth from the second moult through the ninth and, thereafter, there is a gradual decline. This was not only apparent in those analyzed but in all others. Nor is it necessary to have a definite amount of basis growth for a moult to occur; in fact, there are some instances in which no growth of measurable amount occurred but moulting took place. It is possible that shell height increased as well as the size of the animal body enclosed within the mural plates. A satisfactory and accurate method of measuring shell depth and body size could not be devised and thus such increases could not be included in this study.

Conclusions

1. Young *Balanus improvisus* may be reared on uni-algal cultures of *Nitzschia closterium* or *Chlamydomonas* sp. or a mixture of these.

2. *Balanus improvisus* exhibits more rapid growth in a medium containing *Chlamydomonas* sp. alone than in a mixed culture of *Nitzschia closterium* and *Chlamydomonas* sp.

3. *Balanus improvisus* grown in uni-algal cultures exhibit continuous growth but it is less than that found under natural conditions.

4. Barnacles maintained in the Harbor and in cultures show immediately after settling a slow increase in basis size, then a gradual increase which becomes more rapid, followed by a period of reduced growth.

5. The time of moulting and period of intermoult have been determined from the time of settling to 64 days for 57 barnacles in Series I, to 57 days for 100 barnacles in Series II, to 13 days for 10 barnacles in Series III, and to 13 days for 38 barnacles in Series IV, a total of 204 barnacles. A portion of Series II was followed through 120 days.

6. Moulting occurred on the average of every 2–3 days in all series maintained.

7. *Balanus improvisus* reared on *Chlamydomonas* sp. exhibit a more regular time of moulting than those grown on *Nitzschia closterium* or a combination of the two culture media.

8. Shell growth is a continuous but erratic process and there is no indication, from these studies, that there is any correlation between shell growth and moulting.

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THE ROLES OF DARKNESS AND LIGHT IN THE PHOTOPERIODIC RESPONSE OF THE TESTES OF WHITE-CROWNED SPARROWS[1]

DONALD S. FARNER, L. R. MEWALDT[2] AND STANLEY D. IRVING

Laboratories of Zoophysiology, Department of Zoology, State College of Washington, Pullman, Washington

During the past quarter century a substantial amount of investigation has been directed towards the so-called photoperiodic response in birds, whereby it is possible to obtain an unseasonal gonadal development by increasing the length of the daily photoperiod. These investigations have been summarized very adequately by Burger (1949) and Benoit (1950). A very interesting and useful approach to an understanding of the basic mechanism involved in this phenomenon is the use of interrupted daily photoperiods. The first use of this experimental approach was apparently that of Benoit (1936). In his experiments young male ducks were maintained in dim essentially non-stimulatory light except for the experimental periods of exposure to light of stimulatory intensity when they were transferred to a light room. Benoit found that ducks exposed to 10.5 minutes of light per hour for 14 hours per night (ca. 2.5 hours total light) for a period of 16 days showed a generally greater testicular response than those which received single periods of 2.5 hours per night. Burger *et al.* (1942) obtained testicular responses in starlings (*Sturnus vulgaris*) given ten hours of light daily as a period of six hours continuous light plus eight hours of alternating 5-second light and 5-second dark periods. The entire treatment had a duration of 14 hours daily; the testicular responses were the same as those of starlings which received daily 14-hour periods of continuous light. Uninterrupted 10-hour light periods are essentially non-stimulatory in this species.

Kirkpatrick and Leopold (1952, 1953) have recently shown with bobwhite quail (*Colinus virginianus*) that, although 10 hours of continuous light daily for 37 days failed to evoke a gonadal response, the same total duration of light, but with 15–60 minutes used as an interruption of the dark period, resulted in gonadal activation. It was found that the percentage of full sexual activity after 64 days of such treatment was directly proportional to the length of the interrupting light period and inversely proportional to the length of the longer dark period. These results prompted the investigators to conclude that both the light period and the dark period are involved in sexual activation in this species, that the dark period is an inhibiting factor, and that the duration of the dark period is a major controlling factor in the response. Jenner and Engels (1952) in experiments with male slate-colored juncos (*Junco hyemalis*) and white-throated sparrows (*Zonotrichia albicollis*) have

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[2] Present address: Department of Natural Sciences, San Jose State College, San Jose, California.

likewise found that it is possible to obtain testicular development with a total of 10 hours of light per day when 1¾ hours of it were used as an interruption in the middle of the dark period. Ten-hour daily photoperiods are essentially non-stimulatory in these species. These investigators have concluded that in the photoperiodic response of these species there is a (p. 354) ". . . critically important dark-dependent phase." This is somewhat difficult to rationalize in light of the testicular responses obtained by Winn (1951) and Wolfson (1952a) in the same species with exposure to continuous light.

We have recently completed a series of experiments in which we have exposed male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to several patterns of intermittent lighting. The results of these experiments are in accord with the data obtained by Kirkpatrick and Leopold (1952, 1953) and Jenner and Engels (1952); however, they lead us to feel that the assumption of an essential or active role by the dark period in the photoperiodic response is not necessary. It is the purpose of this paper to describe the results of our experiments and to present an hypothesis which rationalizes the available data on avian gonadal responses to intermittent lighting.

Methods

Our experimental birds were captured with Japanese mist nets from the population of *Zonotrichia leucophrys gambelii* which winters in the Snake River Canyon of southeastern Washington. On February 14, after an adequate period of con-

Table I

Resumé of experimental conditions

Group	Number of males	Mean aviary temperature °C.	Daily light treatment		
			Schedules	Total hours of light	Total hours of treatment*
A	8	20	0700–1700 continuous	10	10
B**	12	6	0554–1806 continuous	12.2**	12.2**
C	7	21	0300–2100 continuous	18	18
D	10	16	0800–1630 and 2330–0100	10	17
E	15	20	Continuous 0710 to 1650 plus one minute each half-hour from 0300 to 0700 and from 1700 to 2100	10	18
F	13	21	Nine 1.11-hour periods separated by 1-hour periods of darkness (0300 to 2100)	10	18

* Time elapsed from the beginning of the first (or only) period of light to the end of the last (or only period) of light.

** Wild controls; mean daylight hours February 14 to March 16 including civil twilight.

ditioning to cage life, five groups were placed in separate aviaries and subjected to the experimental conditions indicated in Table I. Additional wild birds (group B of Table I) were collected and examined through the course of the experiments. Each group contained a few females; because of the small numbers and the variability in responses in this sex, the data from these are not included in this report. The

illumination was provided by incandescent lamps at intensities of 30–40 foot candles within each cage. This is substantially in excess of the minimum required to induce a gonadal response. All lights were operated automatically. Groups A and E were kept in separate light-insulated compartments in the same constant-condition aviary. The other three experimental groups were maintained in widely separated indoor aviaries in which thermographic recording was employed to ascertain mean temperatures.

The birds in all experimental groups were kept in flight cages ($12 \times 24 \times 18$ inches), about six per cage. Food, water, and grit were provided *ad libitum*. Food consisted of red millet seed supplemented by a vitamin- and mineral-enriched poultry mash prepared by the Department of Poultry Science of the State College of Washington.

The treatments, as indicated in Table I, were continued for 29–30 days. Body weights and observations on the plumage were recorded weekly. At the conclusion of the experimental period the birds were killed and autopsied. The gonads were placed in alcohol-formaldehyde-acetic acid fixing mixture, and after several

TABLE II

Testicular weights

Group	Number	Arithmetic mean (mg.)	Range	Geometric* mean (mg.)
A	8	1.2	0.6–1.6	1.2
B	12	3.9	3.0–6.0	3.8
C	7	190	79–250	170
D	10	8.2	3.0–25	6 8
E	15	87	17–210	65
F	13	170	35–310	140

* Obtained by logarithmic transformation of testicular weights.

days, to allow thorough impregnation, were weighed on a Roller-Smith Precision Balance. For histologic examination, one testis from each male was imbedded in paraffin, sectioned at eight micra, and stained with acid hematoxylin and yellowish eosin.

We are indebted to Dr. J. G. Darroch for advice concerning the statistical analyses.

RESULTS

The responses to the various treatments, in terms of testicular weights, are given in Table II. Unfortunately it was not possible to maintain the temperature of the aviary containing group D at the same level as that of the other experimental groups. Had this been possible, the response of the testes in group D would doubtless have been somewhat greater (Farner and Mewaldt, 1952) although it is highly improbable that the degrees of development of groups C, E, and F would have been approached. The test of Cochrane and Cox (Snedecor, 1946) for significance of differences between means involving samples of different sizes and differences in variance has been applied to all possible pairs of arithmetic means.

All differences are highly significant ($P = 0.01$) except those between the means of B–D, C–E, C–F, and E–F; however, the differences between the means of C–E and E–F are significant at the 5 per cent level. Statistical analyses of the logarithms (Bartlett, 1947) of testicular weights indicate that the differences between all possible pairs of geometric means except C–F are highly significant.

In our histologic studies we have first compared a normal series of wild and captive birds with the description of testicular development in this subspecies as presented by Blanchard and Erickson (1949). We found their description to fit our material very well, with the exception of the distinction between stages I and II (see also Blanchard, 1941) which is based on the absence of cells of Leydig in the former. We have consequently adopted Blanchard's (1941) stages in the analysis of our material although we do not separate stages I and II. The results of our histologic studies are presented in Table III. It should be noted that the 10-hour controls (group A) showed no development beyond the normal winter resting condition,

TABLE III

Testicular development

Group	Number examined	Stages* of development
A	8	8 in I–II
B	2**	2 in III**
C	7	4 in VI; 3 in VII
D	10	7 in III; 3 in IV
E	15	3 in IV; 5 in V; 5 in VI; 2 in VII
F	13	1 in V; 3 in VI; 9 in VII

* According to Blanchard (1941).

** Of the 12 birds in this group only two were examined histologically. Histologic examination of another and more extensive series of testes from birds collected in the wild reveals that all were in stage III on March 16.

whereas the wild controls, subjected to the normal spring increments in daily photoperiod, had reached stage III by March 16. In general the responses ascertained histologically closely parallel the responses indicated by testicular weights.

DISCUSSION

The nature of the mechanism of the photoperiodic gonadal response in birds is by no means completely understood. That the anterior pituitary gland is an essential part is patent; much of the pertinent evidence has been summarized by Burger (1949) and Benoit (1950). The ingenious experiments of Benoit (1937, 1938a, 1938b; Benoit and Kehl, 1939; Benoit and Ott, 1944) have shown further that nonocular reception of light may be involved, and that the hypothalamus is quite likely an important part of the mechanism. Wolfson (1952a) has shown that the gonadotropic effect of daily photoperiods, at least between certain minimum and maximum lengths, is a direct function of photoperiod length, and that the daily increments appear to be summated in exerting their effect. A consideration of the results of our experiments suggests the possibility that this light-stimulated mechanism is of

such nature that there is involved a process which becomes active almost immediately after the beginning of the photoperiod and which has a persistent "carry-over period" of activity after the end of the photoperiod. We envision the effective duration of this "carry-over period" to be of the order of a fraction of an hour to several hours, doubtless a function of the duration and nature of the preceding photoperiod. We likewise envision its characteristics as differing in different species. Our hypothesis suggests further that the rate of testicular response would be a direct function of the summated daily gonadotropic effects of the photoperiods and the "carry-over periods." Under natural conditions, of course, there would be one of each per day. Although our data provide no direct indication of the possible physiologic basis for such a "carry-over period," several suggestions may be made in order to suggest the plausibility of the hypothesis. It is possible that the actual synthesis of gonadotropins could involve a light-dependent reaction. The "carry-over period" would then be the period of the dissipation of the gonadotropins down to an ineffective level. There is also the possibility that the actual transfer of the pituitary gonadotropins into the blood stream involves a light-dependent reaction. This is in accord with recent evidence of Benoit *et al.* (1950) which suggests that the anterior pituitary of the duck tends to release gonadotropins during light periods and to retain them during darkness. Should this be the case, the "carry-over period" might be the time required for blood gonadotropins to fall below a threshold concentration. There is further the possibility that the light-dependent reaction may be in the hypothalamus where it produces a substance which exerts a humoral control over the anterior pituitary.

With respect to the results of our experiments, this hypothesis can account for the difference between groups A and D as the effect of the additional "carry-over period" in D. The treatment and responses in group D were quite similar to those described by Jenner and Engels (1952) for white-throated sparrows and slate-colored juncos. The differences between D and E could be attributed to a longer period of gonadotropic effect in the latter because of the 17 additional "carry-over periods." The differences between E and F could be the result of the greater activity of a "carry-over period" which follows a one-hour photoperiod than of one which follows a one-minute photoperiod. Further investigations are needed to ascertain more precisely the functional relationship between the activity of a "carry-over period" and the duration of the preceding photoperiod. The data of Kirkpatrick and Leopold (1952, 1953) actually fit this hypothesis quite nicely. The inverse relationship between the length of the longer dark period and the gonadal response might be explained as the attainment of the maximum effects of two "carry-over periods" by optimum timing of the interrupting light. The direct relationship between the length of the interrupting light period and gonadal response, as reported by Kirkpatrick and Leopold (1953), conforms with our suggestion that the activity of a "carry-over period" is some direct function of the length of the preceding photoperiod. We feel that this hypothesis also rationalizes the results obtained with interrupted photoperiod by Benoit (1936) on ducks, and quite possibly also those of Burger *et al.* (1942). In the latter case it would be necessary to assume that very short photoperiods (5 seconds) were followed by "carry-over periods" of effective gonadotropic activity in the order of at least a few seconds duration.

It appears that this hypothesis might also be applicable to the mechanism of light-stimulated increase in egg production in the domestic fowl since it has been shown by Dobie *et al.* (1946), Staffe (1950, 1951), and Weber (1951) that various patterns of interrupted light give greater responses than the same amount of light as a single photoperiod. It appears that the reasoning in our hypothesis may be quite similar to that of Staffe (1951) although he is concerned primarily with the mechanism of the stimulation of increased pituitary activity with respect to maintaining a high rate of egg production. The response of sticklebacks (*Gasterosteus aculeatus*) to intermittent light as observed by van den Eeckhoudt (1947) suggests that such a mechanism may exist in this species. On the other hand it appears unlikely that the gonadal responses obtained in photoperiodic investigations of sheep (Yeates, 1949; Hart, 1950) and ferrets or mink (Hammond, 1951, 1952; Hart, 1951) can indicate such a simple rationalization for the mammals with photoperiodically-regulated reproductive cycles. In this respect it should be noted that Hammond (1952) is of the opinion that the mechanisms are basically different in birds and mammals.

Apparently in most, if not all, avian species a refractory period develops following a cycle of gonadal development. During this refractory period an increase in daily photoperiod does not evoke a gonadal development. The available evidence (Damsté, 1947; Wolfson, 1952a, 1952b) suggests that short daily photoperiods, or long dark periods, are necessary to terminate the refractory period. However, once the refractory period has been terminated, our hypothesis suggests, apparently unlike the thinking of Kirkpatrick and Leopold (1952) and Jenner and Engels (1952), that the daily dark period makes no positive contribution to the mechanism of response, although, of course, the gonadotropic effect of a "carry-over period" may be exerted during the dark period. The differences may be largely semantic; we feel, however, that our hypothesis is basically different and represents a more logical rationalization of the available data. Our hypothesis is then consistent with the suggestion of Hammond (1953) that darkness may have no active role in avian photoperiodism.

Kirkpatrick and Leopold (1952) and Jenner and Engels (1952) have emphasized possible analogies between photoperiodism in birds and in plants on the basis of their conclusions that darkness has some essential role in avian photoperiodism and the rather extensive body of evidence (Leopold, 1951; Lang, 1952) of the existence of dark-dependent reactions important in photoperiodism in plants. We feel, however, that the greatest of caution must be exerted in any serious development of such an analogy on the basis of the presently available information on birds, since there appears to be no reason to assume that the effect of darkness in the photoperiodic mechanism is anything other than that of the cessation of a process requiring light.

The hypothesis of the "carry-over period" is proposed simply as one possible rationalization of the available information on the nature of the mechanism of photoperiodism in birds. Its extension and its ultimate acceptance will rest on the results of future investigations. In consideration of the available evidence, however, we feel that two arguments favor this hypothesis: (1)it rationalizes the available information, and (2) as we have argued in our preliminary report, it is in accord with the Law of Parsimony as the simplest apparent rationalization.

SUMMARY

1. Experiments were conducted with males of a migratory race of white-crowned sparrows to ascertain the nature of response to interrupted photoperiods of several patterns.

2. The results were as follows:

(a) Testes of birds on a 10-hour continuous daily photoperiod showed no response during the 29–30 days of the experiment.

(b) Wild controls, exposed to the naturally increasing daily photoperiods from mid-February to mid-March had just begun vernal spermatogenic development and showed slight increases in testicular weight.

(c) Exposure to continuous 18-hour daily photoperiod resulted in complete spermatogenesis, or nearly complete spermatogenesis, and a marked increase in testicular weights.

(d) Daily exposure to 10 hours total light divided into two periods, 8½ hours during the day and 1½ hours in the middle of the night, resulted in a considerably greater degree of gonadal development than the wild controls.

(e) Daily exposure to 10 hours total light, as 9 hours 40 minutes of continuous light plus one minute light per half-hour for 4 hours before and after the continuous period, resulted in a markedly greater response than in (d).

(f) A still greater response was obtained with daily exposure to 10 hours total light divided into nine 1.11-hour periods equally spaced through the course of 18 hours. Spermatogenesis was as completely developed as in (c). Statistically there is no significant difference between the response in testicular weight of this group and the group subjected to continuous 18-hour daily photoperiods.

3. These results suggest to us that the light-stimulated mechanism involves a light-dependent process which responds quickly at the beginning of a period of light and soon produces a sufficient quantity of an essential substance to allow a persistent gonadotropic effect after the cessation of light. We refer to the period of gonadotropic effect after the cessation of light as the "carry-over period." It is our opinion that the assumption of an active or essential role with respect to the dark period, is not necessary in rationalizing the available information on photoperiodism in birds.

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THE PROCESS BY WHICH THE PUPARIA OF MANY SPECIES OF FLIES BECOME FIXED TO A SUBSTRATE

G. FRAENKEL AND VICTOR J. BROOKES

Department of Entomology, University of Illinois, Urbana, Illinois

It is common knowledge among students of the biology of flies that the puparia of many species are firmly fixed to a substrate. This is easy to observe in laboratory cultures of *Drosophila*. Toward the end of the larval period the larvae leave the substrate to meander for a short period on the walls of the culture bottle. The puparia are always firmly glued to the walls of the vessel. The process by which the puparia of *Drosophila* and other flies become fixed to a substrate does not seem ever to have been described. In this paper we shall demonstrate that the puparia are fixed to a substrate by means of a sticky secretion which emanates from the salivary glands.

It is well known that the salivary glands of *Drosophila* and other flies swell towards the end of larval life, and that a semi-liquid secretion fills the lumen of the glands at that time. In *Drosophila*, this is the stage most favorable for a study of the giant chromosomes of the salivary gland cells. The histological and cytological changes in the cells at the time of this secretion have been studied extensively and the presence of this secretion has been recognized and commented upon by a number of investigators (Bodenstein, 1943, 1950; Mellanby, 1938; Painter, 1945; Hsu, 1948; Blumel and Kirby, 1948; Kodani, 1948; Lesher, 1951, 1952). Some of these authors have searched for a possible function of this secretion, but none of them has recognized, or even hinted at what we consider to be the true function, namely the fixation of the puparium.

The results of this investigation have been briefly reported (Fraenkel, 1952). In this paper the process of fixation and the changes in the salivary glands will be described, while the identity of the protein in the salivary gland and in the puparial glue will be demonstrated elsewhere (Moorefield and Fraenkel, unpublished data).

Material and Methods

Most of the observations and experiments described below were carried out on the blowfly, *Phormia regina*, and on *Drosophila melanogaster*. The process of fixation to a substrate was studied by direct observation of fully grown larvae under a binocular microscope. All dissections were made in a modified Ringer solution, consisting of H_2O, 1000 cc.; NaCl, 7.5 g.; KCl, 0.35 g.; and $CaCl_2$, 0.21 g. Larvae of *Phormia* were dissected by cutting off the last segment of the body and pushing the head inwards with the blunt end of a pin while holding the body with a pair of forceps. The larvae were thus turned inside out. The major difficulty of removing the salivary glands was loosening them from the tracheae which lie in close association with the gland. If the gland was punctured in any way, it collapsed and became opaque. No satisfactory method was found other than cautiously break-

ing these tracheal connections with a pin. *Drosophila* larvae were dissected by submerging in Ringer solution and cutting along the ventral surface with a small piece of a razor blade.

Salivary glands have also been observed in the living insect *in situ* by pressing larvae in water under a cover slip or slide and rolling them back and forth until the glands could be seen under a microscope.

Experiments and Observations

1. Description of the secretion and fixation process

A. Drosophila melanogaster

Towards the end of the third instar the larva leaves the food and moves along the sides of the culture tube. It moves in this manner for about 12 hours, and then the movements gradually slow down until the larva comes to rest. The head continues the brushing movements which have gone on throughout larval life but now this movement describes a semicircle around the thorax. As the body gradually contracts to the barrel shape of the puparium, this movement slows down. Motion now all but stops. The mouth hooks are withdrawn but continue to move slightly back and forth. When the pupal contraction is about completed, the mouth pulsates and a clear fluid suddenly pours from it and flows along the area of contact of the body with the surface. The emission of the fluid is accompanied by a back-and-forth or pumping movement of the mouth parts and a contraction and expansion of the body. The fluid begins to harden almost as soon as it is emitted. Once the secretion is completed, the larva becomes motionless and within 30 minutes the puparium begins to darken.

B. Phormia regina

Fully fed third instar larvae leave the meat and wander about in the dry substrate provided for them, sand or sawdust, for about three days. During this period the crop becomes emptied and very much reduced in size. Several hours before the formation of the puparium the larva becomes more and more sluggish and opaque. Finally the prepupa contracts slowly to the shape of the puparium. A description of the pupal contraction and its underlying processes has been given previously (Fraenkel and Rudall, 1940). By the time the pupal contraction is nearly completed and all forward motion has ceased, the anterior segments of the body alone are still capable of some movement. Slow rhythmic contractions then start in about the fourth externally visible segment and move backwards. The larva ex-

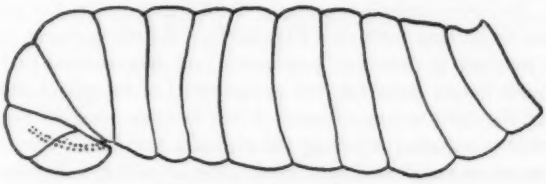

Figure 1. The larva of *Phormia regina* (Meig.) in the act of secreting.

trudes its proboscis, curves it toward the anterior ventral surface (Fig. 1), and then alternately inverts and extends the mouth hooks, working back and forth in motions not unlike the normal feeding motions, but directed toward its own under-surface. At the same time a pumping motion of structures within the head is visible. After the above described motions have continued for a few seconds, a clear colorless fluid emanates from the mouth and is distributed over the anterior under-surface by moving the head from side to side and back and forth in a painting or brushing movement. The fluid is tacky almost immediately after emission and hardens within a few minutes. The anterior ventral surface becomes thus firmly fixed to a surface. The time interval of actual secretion was no longer than thirty seconds.

When larvae are allowed to pupate in sand or sawdust the process of secretion is exactly the same, and in consequence clumps of sand grains or flakes of wood become firmly stuck to the anterior ventral surface. In an overcrowded culture, puparia often become stuck to each other.

Immediately after completion of this process the anterior end is permanently withdrawn into the body and the formation of the white puparium is completed. Darkening starts 15 to 30 minutes later.

2. The salivary glands of *Phormia regina*

One of the major difficulties in following salivary gland development is determination of the maturity of the larva. In this study, the size of the larva, and the amount of the contents of the crop were used as indication of development. The salivary glands of *Phormia* are similar to those described for other fly larvae. They are paired sack-like structures, elongated, extending at full size into the second abdominal segment and connected to each other at the posterior end by a small mass of fat cells.

At the time the larva leaves the food and the crop is still greatly extended with food, the salivary glands are large, distended and clear (Fig. 2a). The cells are stretched, the nucleus is distinct and the cytoplasm clear with a very fine granular consistency. The cells throughout the gland are more or less uniform in size. As the gland gets older the cells become more dense and this accounts for a milky appearance. In older glands the cytoplasm appears to shrink and a clear area is present around the cell walls.

About twenty hours after the larva leaves the food the glands begin to show a narrowing of the lumen in the posterior region (Fig. 2b). As the lumen becomes smaller, the distension of the gland in the anterior region begins to increase, the gland gets longer and begins to have a milky opacity (Fig. 2c–d). As the larva approaches the time of pupation the posterior lumen of the gland becomes more and more occluded, the cells indistinct and the fluid localized in the anterior portion, causing this area to become bulbous (Fig. 2e–f). At the moment that the larva is performing the motions as described previously and diagrammed in Figure 1, about one or two minutes before secretion, the greater part of the gland shows little if any lumen and all of the fluid is concentrated in the swollen anterior region (Fig. 2g). It is this fluid that is expelled, marking the end of larval life.

Figure 2h represents a gland immediately after secretion. The lumen has disappeared almost entirely, the cell walls have become indistinct and the whole struc-

ture is now opaque. Only a small region at the anterior end may still be full, transparent and inflated.

There are many deviations from this scheme, some of which can be explained on the basis of amount of food eaten by the larva. Underfed larvae have glands of normal length, but the lumen is small and the glands, even before the formation of the puparium, resemble somewhat those of well fed individuals after the secretion.

To determine how much the volume of secretion varies in different larvae, ten late third instar larvae were placed in each of ten petri dishes which contained fine clean sand. When all had pupated, they were collected and the amount of sand

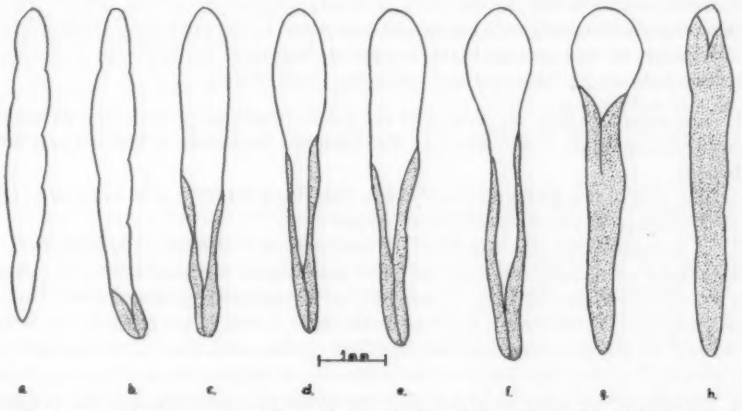

FIGURE 2. The development of the salivary gland of *Phormia regina* (Meig.) from the end of the feeding period to the formation of the white pupa. a, The gland of a 5-day old larva shortly after leaving the food. The lumen is distinct and full of fluid throughout the length of the gland. b, About 24 hours later. The lumen is beginning to disappear in the posterior region. c–d, Later in the sixth day. The closing of the lumen is more pronounced and there is a slight swelling of the anterior portion. e–f, The beginning of the seventh day, several hours before pupation. g, Immediately before secretion. h, The gland immediately after the puparium has become fixed to the substrate.

particles stuck to the surface compared. If sand particles covered more than 50% of the ventral surface or were in large clumps on the head or thorax the secretion was considered large. When only three or four sand grains were stuck to the body the secretion was considered small. The majority of larvae showed sand particles somewhat between these two. The choice is not as arbitrary as it might seem because the differences were very distinct. The variation in the amount of secretion of 100 larvae was as follows:

Volume of secretion	large	average	small	none
Number of larvae	29	48	17	6

The secretion of underfed larvae was usually small or absent.

In another experiment, the salivary glands both of large well fed and underfed larvae were first observed in the living larvae through the cuticle. Well fed larvae

nearly always had large, clear and swollen glands, while those of underfed larvae were not distended and quite opaque. Fifty-four small larvae with empty glands and 24 large larvae with distended glands were then placed in a series of petri dishes which contained sand. Of those with unswollen glands, 10, or 19%, secreted large amounts, while 15, or 62%, of those with swollen glands secreted large amounts. However, only three of the larvae with deflated glands emitted amounts comparable to those with swollen glands.

Discussion

From the observations so far reported it can only be concluded that a secretion which accumulates in the lumen of the salivary gland toward the end of the larval period is expelled immediately before the formation of the puparium is completed and that by means of this secretion the puparium becomes firmly fixed to a substrate. These conclusions are based on the following observations:

1. The secretion into the lumen of the gland develops prior to the formation of the puparium and has disappeared by the time the puparium is formed and fixed to a substrate.
2. The size of the gland as observed in the living larva *in situ* coincides with the relative volumes secreted by different larvae.
3. In a subsequent investigation (Moorefield and Fraenkel, unpublished data) a comparison was made between the total amino acid composition of the secretion from the lumen of the salivary glands and of the puparial glue collected from fully formed puparia. The same 15 amino acids were found in the proteins of both materials, and in addition glucosamine and free lysine, and the two materials proved to be identical within the limits of the paper chromatographic techniques employed.
4. Finally, if we were to argue that the prepupal secretion did not originate in the salivary glands, what other organ could be offered in their place? In fly larvae there are only two other organs large enough to harbor the amount of fluid which is ultimately discharged, the crop and the mid or hind gut. The crop becomes almost entirely reduced prior to the formation of the puparium and what little material occasionally remains is highly colored. Just before puparium formation the larva empties the remaining contents of the intestine through the anus. Thus by exclusion, we are again led to the salivary glands.

The enormous increase in size of the salivary glands during, and especially toward the end of the third larval instar has been observed by Ross (1939), Bodenstein (1943), Painter (1945), Hsu (1948) and Lesher (1951). Some of these authors ascribe this growth to the accumulation of secretory substances, which have been variously described as secretory globules (Ross, 1939; Painter, 1945) or deutoplasmic substances (Lesher, 1951). At the time of formation of the puparium these globules have largely disappeared from the cytoplasm of the cells (Painter, 1945). Bodenstein (1943) objected to an interpretation of these cellular inclusions as a true secretion, since he could not see a function for such a secretion at a time when feeding has completely ceased. Instead he claims these changes to be the result of a beginning histolysis.

Hsu (1948) and Lesher (1951) closely followed this argument. They recognized the passing out of these granules and their accumulation in the lumen of

the salivary glands and ascribed it to the histolysis of the cells. Hsu interpreted the function of this material as that of food storage. Lesher (1952), on histochemical evidence, interpreted these substances as a conjugated protein of the nature of a mucopolysaccharide, with carbohydrate bonded to protein, and suggested its function to be that of a chitin precursor.

Kodani (1948), following earlier work by Blumel and Kirby (1948) recognized that the enlargement of the salivary glands was due to the appearance of material in the lumen of the gland. He collected the accumulated material, recognized its proteinous nature and identified by paper chromatography 15 amino acids. According to his description, the secretion appears in the lumen and later disappears. He finally suggested that it might form part of the pupal body or participate in chemical processes of histolysis. All these authors seem to have been confused and misled by the task to describe and interpret the appearance of what undoubtedly looked like the product of a secretory activity at a period when all feeding has ceased and no such activity was expected to occur. The ultimate function of this secretion as the glue which fixes the puparium to a substrate finally disposes of this dilemma.

All the evidence presented so far in this discussion was concerned with *Drosophila*. Comparable investigations are so far lacking for other flies, although there is little doubt that similar cycles of secretion may be assumed to exist there. Mellanby (1938) has commented on the enormous size which the salivary glands reach in the diapausing prepupae of *Lucilia sericata*. He interpreted this phenomenon as a storage of water, an assumption which is entirely devoid of evidence.

The particular mechanisms of the formation of the puparium in cyclorrhaphous flies is a phenomenon singular to this suborder of Diptera. The formation of a glue by the salivary glands, to fix the puparium to a substrate, therefore could not be expected to have a close analogy among other suborders of Diptera. In a more general sense, however, secretion of proteinous substances by salivary glands, the best known examples of which are the spinning of threads often to form a cocoon, are widely distributed phenomena in many orders of insects. Amongst Diptera, spinning occurs frequently in representatives of the suborder Nematocera. In Chironimidae, the larvae reinforce the walls of the tubes they form in mud by a secretion which emanates from the salivary glands (Pause, 1919), a fact which may account for the exceptionally large size of the salivary gland cells. The spinning habits of several representatives of mycetophilids have been well described (Medwar, 1935; Fulton, 1939, 1941). In *Ceroplatus testaceus* Dalm. spinning has been shown as the activity of well developed salivary glands (Stammer, 1932). Spinning also occurs among representatives of the family Simuliidae (Fulton, 1939).

It is not suggested by the authors that the formation of a puparial glue is the only function of the salivary glands of fly larvae. The production of digestive enzymes in the salivary glands during the feeding period has always been assumed, but has been demonstrated only in a few cases. However, some function other than that described in this paper, must be postulated for the salivary glands, especially in view of the fact that the puparia of many species of flies never are fixed to a substrate. This is the case with *Musca domestica, Calliphora erythrocephala, Sarcophaga falculata* and *S. crassipalpis*. Fixation has so far been observed by the writers in several *Drosophila* species, in *Lucilia sericata, Phormia terra-novae* and *P. regina*. It

is indicated from a few preliminary observations that the salivary glands of species of flies which do not have this secretion, do not show the spectacular enlargement towards the end of larval life, as found in the other group. A comparison of the activities of salivary glands of many species of fly larvae would appear to be an interesting and necessary subject of study.

Some of the observations recorded in this paper were made by Mrs. E. Lichtwardt while working in this department. The authors are indebted to Dr. Betty Walshe, Bedford College, London and Dr. John B. Buck, National Institutes of Health, Bethesda, Md., for suggestions concerning the spinning habits of Chironomidae and Mycetophilidae.

Summary

1. The puparia of many flies become fixed to a substrate by means of a secretion which accumulates in the lumen of the salivary glands toward the end of the larval period and is expelled immediately before the formation of the puparium is completed.

2. The process of fixation has been described for *Drosophila melanogaster* and *Phormia regina*.

3. The changes in the size and appearance of the salivary glands of *Phormia regina* during the accumulation of the salivary secretion and after its elimination have been described.

4. The process of secretion in the glands of *Drosophila* has been previously described by other authors, but its true function has never been recognized.

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SYNTHESIS OF PIGMENT DURING THE RECONSTITUTION OF TUBULARIA [1]

ALLEN S. GOLDMAN [2]

Arnold Biological Laboratory, Brown University, Providence, R. I.

During the reconstitution of the hydroid, *Tubularia,* a red pigment appears in the region where the new hydranth structures will arise (Loeb, 1891; Driesch, 1900; Morgan, 1901; Stevens, 1901, 1902). This pigment is presumably the carotenoid, astacene, which gives the characteristic red color to the adult hydranth and the pink color to the adult stem (Lönnberg and Hellström, 1931; Karrer and Jucker, 1950; Cohen, 1952). Little is known concerning the origin of the pigment during reconstitution, but previous work has suggested four possibilities (*cf.* Cohen, 1952).

1) The pigment could be synthesized by symbiotic red algae which are believed to occur occasionally in *Tubularia.* This is an appealing possibility because no animals are known with certainty to synthesize carotenoids (*cf.* Karrer and Jucker, 1950).

2) Pigment in the gastrovascular fluid could be taken up differentially by the reconstituting region. Algae or small crustaceans, which are regularly ingested, could provide the source of this pigment, or it could be extruded from cells located at some distance from the forming hydranth.

3) Pigment-containing cells from other regions of the stem could migrate into the hydranth rudiment. This possibility is favored by the observation (Cohen, 1952) that the coenosarc thickens in the regenerating zone and becomes thinner in adjacent regions. Tardent's (1952) finding that the interstitial cells of the ectoderm can migrate extensively also indirectly supports this alternative.

4) Finally, new pigment could be synthesized in the coenosarc, either *in situ* or throughout the stem. If the synthesis were not localized in the reconstituting area, newly formed pigment might then be redistributed according to alternatives 2 or 3.

A study of reconstitution in short segments of stems should permit a choice among the various origins of pigment outlined above. A reconstituting segment becomes completely enveloped by perisarc and does not feed. Although reconstitution will occur in segments of any desired length, the new hydranth actually develops in a region only about two millimeters long. Segments of minimal length are almost wholly involved in hydranth formation. Therefore, by using short segments, it should be possible to determine whether a synthesis of pigment occurs or whether some source of pigment extrinsic to the regenerating segment is essential.

[1] This paper is a revision of a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biology at Brown University, Providence, R. I.

[2] Present address: Osborn Zoological Laboratory, Yale University, New Haven, Conn.

The following account presents the results of such a study in which both histological and spectrophotometric methods were utilized. The data obtained prove that new pigment is synthesized by the coenosarc of a reconstituting segment.

The author is grateful to Dr. Edgar Zwilling for suggesting this problem and especially to Dr. Mac V. Edds, Jr. under whose guidance the work was carried out. Acknowledgment is also due to Dr. Paul F. Fenton and to Dr. William Montagna for their assistance with the technical aspects of this investigation.

Materials and Methods

Colonies of *Tubularia crocea,* furnished periodically by the Supply Department of the Woods Hole Marine Biological Laboratory from August, 1952 to January, 1953, constituted the experimental material. Experiment 1 was begun at the Marine Biological Laboratory and completed at the Arnold Biological Laboratory. For all other experiments the colonies, freshly collected at Woods Hole, were placed in a thermos jug containing filtered sea water at the temperature of local waters and were transported by automobile to the Arnold Biological Laboratory. Experiments were started within six to eight hours after collection since it was found that the animals autotomized their hydranths and began to decay within 24 hours when not in running sea water.

Handling of living material

Translucent, young, and healthy stems with little or no branching were selected for uniformity of cross-sectional diameter and coloration. Stems with a caliber of from 0.5 to 1.0 mm. were cut into segments from 1.5 to 2.0 mm. in length. Six hundred to 1000 such segments were allowed to reconstitute in three or four large finger bowls, each containing about 100 cc. of filtered sea water. In Experiment 1, begun at Woods Hole, the segments reconstituted at room temperature (20-22° C.); in all subsequent experiments, the temperature was kept between 12 and 15° C.

Reconstituting segments were analyzed at each of four arbitrarily designated phases of the regenerative process. Approximately one quarter of the segments was removed from the culture bowls at each of the four phases. In the latter three phases only segments with visible indications of regeneration were selected for analysis. The segments were placed on filter paper and rolled individually to remove any pigmented algae or actinulae larvae which might be adhering to the perisarc. After a period of drying in air at room temperatures which were relatively constant during the experiments, the segments were weighed. The segments of Experiment 1 were weighed one to two hours after removal from the culture bowls; those of Experiment 2, within 20 to 40 minutes after removal. To minimize the variation in wet weight due to evaporation during shorter drying periods, the time interval from the removal of the first segment to the weighing was standardized at 35 minutes in all subsequent experiments. A period of 35 minutes was selected after a preliminary comparison of weights determined after 35 minutes of drying in air, with weights determined after over-night dehydration at 110° C., demonstrated a good correlation between them. During the last 10 minutes of the drying period, the seg-

ments were placed in the analytical balance chamber to permit the atmosphere in the chamber to come to equilibrium.

Spectrophotometric analysis

The amount of pigment in the reconstituting segments, was determined with a Coleman Junior Spectrophotometer. After weighing, the segments in a given phase of regeneration were transferred to a test tube containing 2 cc. of acetone for extraction of the pigment. The segments were ground with the flat end of a glass rod until most of the visible red color had been dissolved (ca. 5 minutes). The mixture was then stored for an extended period at 12° C. (4 to 6 weeks in Experiment 1; two days in Experiments 2–6) to extract any remaining pigment.

In Experiment 1 after acetone extraction, each acetone-extract, plus a 0.5-cc. acetone wash of the residue, was directly diluted to 5 cc. in a volumetric flask; optical densities were then determined. Since all of the optical densities obtained in this experiment were below 0.05 optical density units, a micro-method was employed in all subsequent analyses (Experiments 2–6).

In this procedure, after extraction with acetone, each acetone extract, plus a 0.5-cc. acetone wash of the residue, was transferred to another test tube and evaporated to dryness (overnight vacuum desiccation). The residue of the once-extracted segments was re-extracted for two days with 2 cc. of acetone. This supernate, plus a 0.5-cc. acetone washing of the residue, was desiccated in a similar manner. The first and second desiccated extracts were then taken up separately in acetone delivered from calibrated volumetric micro-pipettes. A 0.3889-cc. micro-pipette was used in Experiment 2; a 0.1565-cc. micro-pipette in Experiments 3–6. The pigment solutions were transferred to cork-stoppered, standardized micro-cuvettes made from No. 5 Pyrex tubing for the Coleman Junior instrument. To minimize evaporation, all dilutions were carried out in the open freezing compartment of a refrigerator, using previously chilled acetone and equipment. Optical densities were then determined.

The maximum absorption of the extracts fell between 460–470 mμ (*cf.* Cohen, 1952); their optical densities were therefore compared at 470 mμ. The absorption values of the first and second extracts were added, and their sum divided by the wet weight of the segments originally extracted. Since the dilutions employed in Experiments 1 and 2 were different from those used in Experiments 3–6, a dilution factor was introduced to make all absorptions directly comparable.

Histological methods

For histological study of pigment distribution, tissues were fixed in a solution of three parts absolute alcohol-one part acetic acid, and were then sectioned in paraffin and stained with iron hematoxylin. Pigment distribution was also examined in squash preparations of living material viewed either with polarized or white light.

The sudanophilia of adult hydranths and of several reconstituting segments was analyzed, using Baker's (1944) method with the following modification: the tissues were fixed for 20 minutes in a solution containing 10 cc. of formalin, 10 cc. of 10% calcium chloride and 80 cc. of filtered sea water. After three rinses in tap water, the pieces were imbedded in 25% gelatine and cut at 10 μ with the freezing microtome; sections were stained with Sudan black B for 8 minutes.

Experimental Results

The phases of reconstitution selected for study are shown diagrammatically in Figures 1a–1d. The *zero point phase* (Fig. 1a) includes pieces selected immediately after the last segments had been amputated at the start of a given experiment; from 0 to 1½ hours had elapsed since the segments had been isolated and most of their

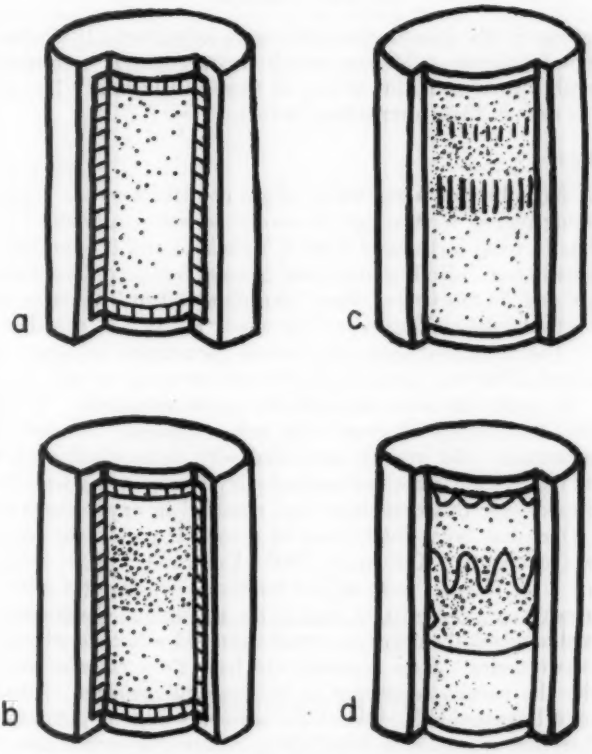

Figure 1. Three dimensional representation of reconstituting *Tubularia* stem segments. The characteristic red pigment distribution is shown by stippling. The clear outer covering represents the perisarc and the hatched layer in a and b, the ectoderm. a, Zero point phase segment showing pigment in the exposed endoderm. b, Condensation phase segment with band of pigment in the future hydranth endoderm. c, Striation phase; the proximal and distal pigmented striations are visible through the intact ectoderm (within the band of pigment). d, Intraperisarcal hydranth phase segment showing the unemerged hydranth within the perisarc.

cut ends had already healed. The first visible indication of reconstitution appears at the *condensation phase* (Fig. 1b) after 34–35 hours at 12–15° C. At this time, a circumferential band of pinkish-red pigment, some 0.5 to 2 mm. in length, becomes localized in the future distal half of the segment. After approximately 40–45 hours

of reconstitution, the *striation phase* (Fig. 1c) can be distinguished. This phase is characterized by two parallel rows of well-defined, longitudinal red striations which have developed in the pigmented band. The final, *intraperisarcal hydranth phase* (Fig. 1d), which appears after about 60–62 hours, features a constriction separating the newly formed hydranth from the coenosarc.

Origin of pigment

As pointed out in the Introduction, the work of Cohen (1952) has suggested that the externally visible reddening which occurs during reconstitution in the area of hydranth formation is due to one of four possibilities. The following account considers each of these alternatives in turn.

Role of red algae

Ceramium and other red algae which might contribute to the reddening of the hydranth-forming region, were sought in several squash preparations of living material as well as in fixed sections of 7 adult *Tubularia* and 8 segments in different phases of reconstitution. Both solitary and colonial forms of *Ceramium,* as well as *Achrochaetium* and *Rhodochorton,* have been observed in *Tubularia,* either in the endoderm or between the endoderm and the ectoderm (W.R. Taylor, personal communication). The individual algal cell is small, rectangular in shape, and contains a readily distinguishable red pigment which is not removed by ordinary histological procedures. No such cells were demonstrable in the coenosarc. Cohen (personal communication) has already observed that only occasional *Tubularia* are infested with these organisms. His results, combined with those obtained in the present study, indicate that red algae are not indispensably related to pigment formation.

Further support for this conclusion was obtained in experiments with $CuSO_4$, a compound which has been widely used as a specific poison for fresh water and marine algae (Moore and Kellerman, 1905; Domogalla, 1926; Prescott, 1938). In experiment 4, the colonies were dipped for about two minutes into a solution of 5% $CuSO_4$ in sea water immediately prior to the amputation of segments. The bottom of the bowl to which the colonies were transferred was covered with dead algae within one to two hours. Stem segments cut from these colonies nevertheless regenerated with the normal reddening of the hydranth anlagen. Identical results were obtained in Experiment 5, in which the segments were amputated 10 days after the colonies had been treated with 5% $CuSO_4$. This observation also ruled out the possibility that extra-perisarcal algal contamination plays a role in pigment formation.

Concentrating of pigment formed elsewhere

According to this view, pigment circulating in the gastro-vascular fluid is taken up by endodermal cells of the reconstituting region. This pigment would be derived either from ingested substances or from cells lying at some distance from the reconstituting region.

This alternative is ruled out by the fact that in the present experiments, reconstitution occurred in short segments which were entirely enveloped by perisarc. They were therefore completely isolated systems with respect to acquisition of new pigment from the rest of the stem, or from feeding activities.

Migration of pigmented cells

This alternative suggests that pigmented cells may migrate from a distance into the reconstituting region. Extensive cell migration cannot be critically important, however, because pigment accumulates normally during the reconstitution of short segments which are isolated from the rest of the stem.

Localized synthesis of pigment

The above observations show that the reddening in the hydranth anlagen cannot be attributed to red algae, ingested materials, or a concentrating of pigment already present. Therefore, the reddening must be due to an increase of pigment within the

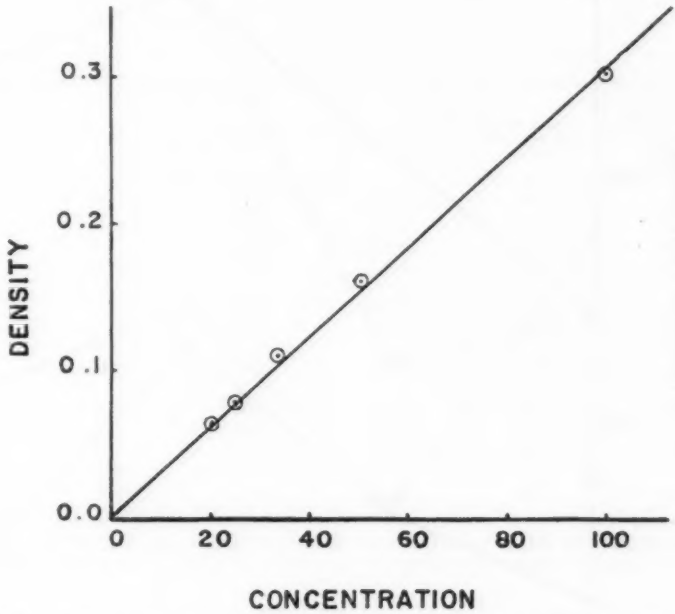

FIGURE 2. Comparison of concentration and optical density of red pigment extracts. Density is plotted along the ordinate as optical density units and concentration along the abscissa as per cent of the most concentrated.

reconstituting segment, *i.e.*, a synthesis of pigment by the coenosarc. Since microscopic observation is not adequate to provide direct evidence on this point, the pigment content of segments at successive phases of the reconstitutive process was determined by a spectrophotometric assay.

Evaluation of method. In order to determine the validity of using optical density as a direct measure of pigment concentration, and the reliability of using the densities obtained in the lower range of the spectrophotometer, the following preliminary determination was made.

A concentrated acetone extract of adult hydranths was prepared and diluted: 1 : 2, 1 : 3, 1 : 4, and 1 : 5. The absorption values for the concentrated extract and the four dilutions were plotted against dilution expressed as per cent of the most concentrated. The resulting curve (Fig. 2) demonstrated that for optical density values of 0.05 or above, a linear relation exists between concentration and density.

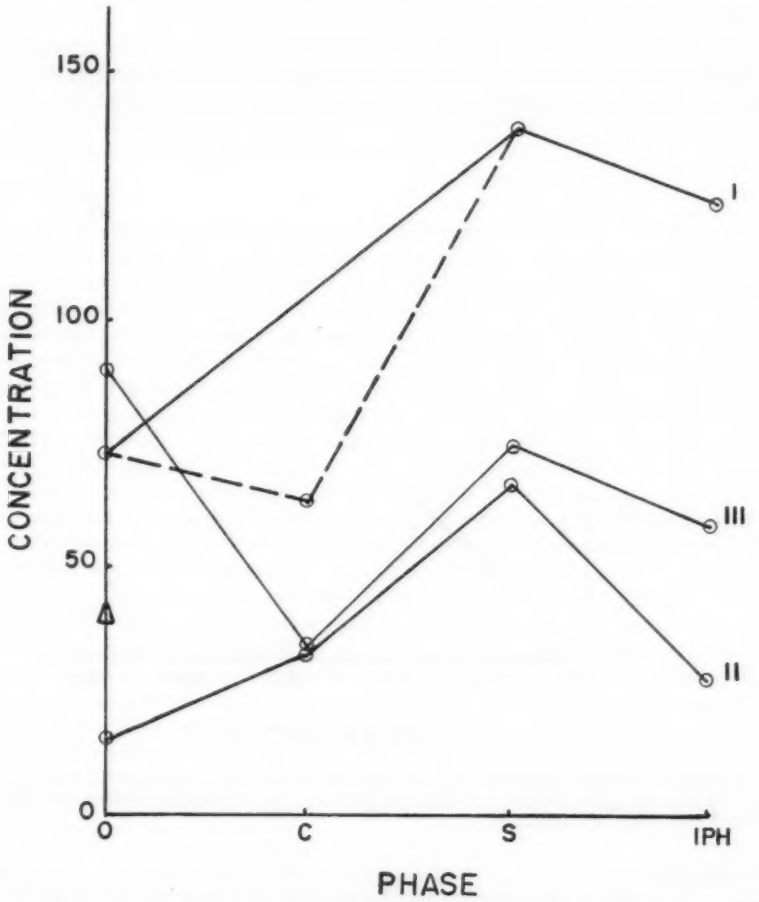

FIGURE 3. Changes in the concentration of red pigment during reconstitution. Concentration, expressed as optical density units per mg. wet weight of segments $\times 10^{-4}$, is plotted against phase of reconstitution. O, C, S, and IPH represent the zero point, condensation, striation, and intraperisarcal hydranth phases, respectively. The optical densities were measured at 470 mμ. The triangle denotes the concentration of red pigment in segments from Experiment 6 which failed to reconstitute after four days of culturing. Curves I, II, and III represent Experiments 1, 2, and 6, respectively.

Several of the values subsequently obtained with the experimental material fell between 0.01 and 0.05 optical density units. These values appear to be trustworthy, however, since curves plotted from them were corroborated in other cases where higher values were obtained.

Spectrophotometric results. The data from Experiments 1, 2, and 6 are shown in Figure 3 (curves I, II, and III, respectively), plotted as pigment concentration per unit wet weight against phase of reconstitution. The values on which these curves are based appear in Table I.

In general, the curves show a marked increase in the amount of pigment from the zero point phase to the striation phase, followed by a moderate decrease from the striation phase to the intraperisarcal hydranth phase. Although the conditions un-

TABLE I

Red pigment extracts

Experiment	Stage*	Wet weight mg.	Optical density	Optical density/ mg. wet weight $\times 10^{-4}$	Optical density/ mg. wet weight at dilution of Exps. 3–6 $\times 10^{-4}$
1	O	44.4	0.012	2.3	73.2
	C	44.8	0.010	2.0	63.7
	S	74.1	0.038	4.3	137.2
	IPH	89.8	0.039	3.9	124.2
2	O	44.8	0.028	6.3	15.8
	C	45.6	0.060	13.2	33.0
	S	35.2	0.096	27.3	68.3
	IPH	36.6	0.040	10.9	27.3
6	O	66.8	0.580	86.8	86.8
	C	96.4	0.327	33.9	33.9
	S	34.0	0.252	74.1	74.1
	IPH	40.2	0.234	58.2	58.2
	N–R	33.6	0.128	41.7	41.7

* O represents the zero point phase; C, the condensation phase; S, the striation phase; IPH, the intraperisarcal hydranth phase; and N–R, the non-reconstituted phase segments in Experiment 3.

der which the data were obtained preclude precise quantitative statements about the absolute increase in pigment, the curves do reveal the approximate range of this increase. They show: 1) that the concentration of pigment increases up to the development of the third phase of reconstitution to values some two- to four-fold above that of the base level of the zero point phase, and 2) that pigment decreases during the development of the last phase to a final value some one- to three-fold greater than that of the base level.

The upward displacement of curve I is probably due to the large dilution factor required to make the original optical density values directly comparable with those comprising curve III (see Materials and Methods). The first three points of curve I are interconnected with dotted lines since the data for the condensation phase in this experiment were obtained from an extract which was recovered after some spill-

age. The second point therefore represents at least a minimum concentration for this phase but the actual value should be higher.

The extract of the zero point phase in Experiment 6 (curve III) was observed to be redder than those of the other phases before desiccation. This indicated that the aberrantly high pigment concentration found for this phase was not due to some procedural error during dilution or spectrophotometry. The high value may be due to either: 1) a non-random selection of more highly pigmented segments to represent the zero point phase, or 2) a decrease, after the removal of zero point phase segments, in the number of pigmented larvae or algae adhering to the remaining segments. Statistically, the first alternative appears unlikely. But there is no direct evidence favoring the second and the issue must be left undecided.

Overlooking the problematical high zero point value of curve III, the graphs in Figure 3 demonstrate that the reconstituting segments gained pigment during the development of the second and third phases and lost pigment before the last phase. These findings are indirectly supported by the observation that there is a much smaller increase in pigment content in non-reconstituting segments. Regeneration of all the segments in a culture bowl never occurred. Therefore, many non-reconstituted segments were available for analysis. The pigment concentration was measured for approximately 200 segments (from the lot used for Experiment 6, curve III) which had not reconstituted after four days. The extract of these segments had an adjusted density value (triangle in Fig. 3) somewhat below that of the aberrant zero point phase of curve III but above the more reliable zero point level predicted both by curve II and the slope of curve I. On the basis of this limited evidence, it is possible that a small increase in pigment content has occurred but in any event the increase amounts to less than half of that exhibited by reconstituting segments.

Taken as a whole, the results of these experiments show that there is an increase in total pigment content of short reconstituting segments. Since the new pigment is not derived from extraneous sources, the coenosarc of the segment must synthesize pigment during the regeneration of a hydranth.

The distribution of pigment. To obtain information about the site of synthesis within the segment, the distribution of pigment in adult and reconstituting individuals was studied histologically. While these studies did not provide a definitive solution, they did reveal some pertinent facts about the form and the location in which pigment occurs. In general, two forms of pigment can be identified: 1) large (2.6–10.3 μ) granules, irregular in shape and red in color when viewed in white light, but appearing as elongate birefringent crystals when seen in polarized light, and 2) minute ($< 1\ \mu$) anisotropic crystals, white and luminescent in polarized light and dark red in white light.

Large pigment granules circulate passively in the gastro-vascular fluid of adult, living *Tubularia;* the large granules also occur intracellularly in the endoderm. In the stem, pigment granules are evident particularly in large, oval cells which are sparsely scattered through the endodermal ridges and the rest of the endoderm. Pigmented material occurs in all endodermal cells of the hypostome and is so concentrated that it is not possible to distinguish individual granules. Presumably, some of the pigment in this region is also in granular form. Since the endoderm phagocytizes pigment from the circulation (Cohen, 1952), the large intracellular granules may have this origin.

Other endodermal cells which do not contain the pigment granules, as well as the heavily colored cells of the hypostome, are filled with small, anisotropic pigment crystals. In each such cell, the crystals are homogeneously distributed and are present in large numbers. Presumably, they account for most of the gross red coloration of *Tubularia*.

Stevens (1901) also described two forms of pigment: 1) translucent, yellowish-red (1 to 8.5 μ) "plasmatic granules" which are insoluble in alcohol, and 2) small, irregular grains of red pigment which are soluble in alcohol. In some endodermal cells, Stevens found both types occurring together; in other cases, only the small grains were present. The large red granules, observed in the present investigation, appear to correspond with Stevens' "plasmatic granules"; the minute anisotropic crystals are probably identical to her irregular grains.

During reconstitution, the intracellular pigment granules appear to remain constant in number. The reddening of the hydranth anlagen is therefore probably due to an increase in concentration of the pigment crystals. It is not clear, however, whether the pigment crystals are formed *in situ* or whether there is a general synthesis in all endodermal cells of the reconstituting segment followed by a concentration of pigment or pigmented cells in the reddening area (see Introduction, alternative 4). Only further study can answer this question.

Distribution and concentration of sudanophilic substances

During the course of the experiments just described, attention was drawn to the possibility that lipid substances might be related to reconstitution. A liquid was noticed which adhered to the sides of the test tubes containing the desiccates of the first pigment extracts of Experiments 2–6. The liquid could not be evaporated by vacuum desiccation for two days. The desiccates of the zero point phases contained the largest amounts of this liquid (presumably lipid), while those of the three subsequent phases contained progressively smaller amounts. This observation prompted a histochemical analysis of the sudanophilia of reconstituting segments. The variations in the degree of sudanophilia which were detected in this analysis could not be related to the relative amounts of liquid in the desiccates. But they provided some important additional information about the reconstitution process. The methods used and the results obtained were as follows.

Studies were made on 4 to 8 sample sections (stained with Sudan black B) of three adult hydranths and stems, and of one or two segments in each phase of reconstitution from Experiments 3 and 6. The degree of sudanophilia was estimated by averaging arbitrarily assigned values for the intensity of coloration of the various regions. Five categories (0, + 1, + 2, +3, and + 4) of sudanophilia, representing a range from the least to the most intense coloration, were distinguished. In most cases it was possible to assign a value of 0, + 2, or + 4; the intermediate values of + 1 and + 3 were required only in a few instances. The average level of sudanophilia in each of the various regions of each phase was plotted against the phase of reconstitution (Fig. 4).

Adult hydranths and stems exhibit two distinct levels of sudanophilia. The endodermal cells of the hypostome are intensely colored (+ 4) while the stem endoderm contains smaller, diffusely distributed sudanophilic globules. The degree of coloration is similar (+ 2) in the endodermal ridges, in the endoderm subjacent

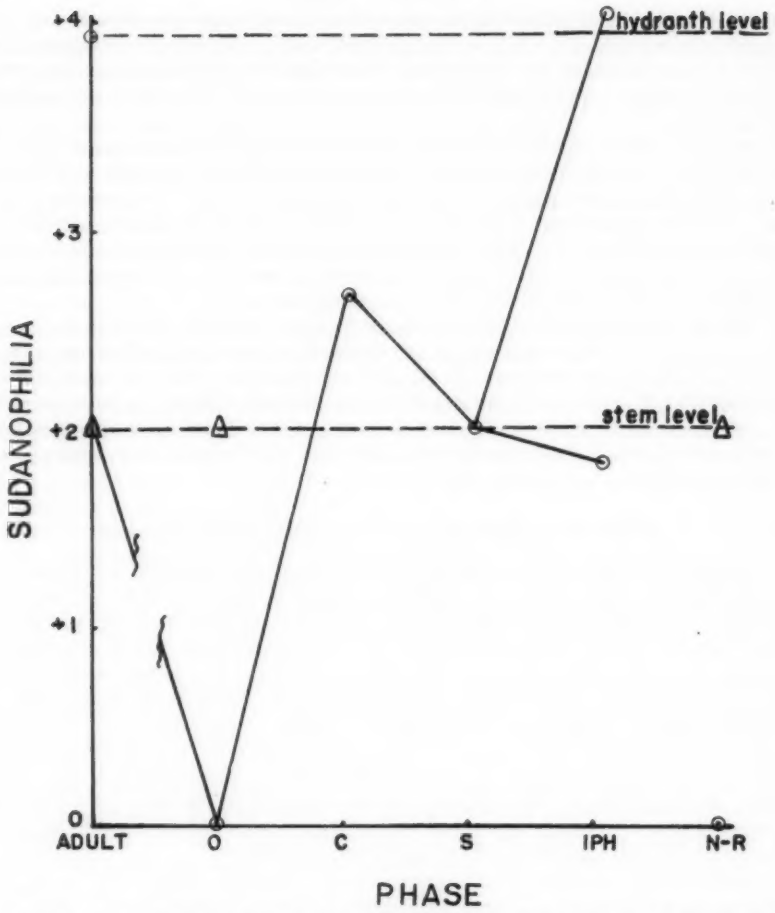

FIGURE 4. Changes in the degree of sudanophilia during reconstitution. Sudanophilia is plotted as arbitrarily designated levels of staining intensity (see text) against phase of reconstitution. N–R represents segments which had failed to reconstitute after four days of culturing; other abbreviations are as in Figure 3. Circles indicate values for the circumferential endoderm; the triangles, those for the endodermal ridges and their subjacent endoderm. The curve from the adult to the zero point phase is broken because the adults were not examined at a time in sequence with the regeneration process.

to the ridges, and in the rest of the endoderm (except for the oval cells which are sudanophobic). The pigment crystals are found in close association with some of the sudanophilic globules.

Two levels of sudanophilia also characterize the endoderm of the zero point phase segments. The cells of the endodermal ridges, which are intact only in some

instances, and the subjacent endoderm, maintain a + 2 level of sudanophilia. The rest of the endoderm has dropped to a zero level. The endodermal ridges in the reconstituting end, and in some cases in both ends, break down before the appearance of the condensation phase (Morgan, 1901; Stevens, 1901, 1902); in this phase, the sudanophilia of other endodermal cells has increased to approximately a + 2 level. There is no significant subsequent change in endodermal sudanophilia up to the striation phase. Thereafter, however, a differential distribution of sudanophilic substances is evident. In the intraperisarcal hydranth phase the + 2 intensity of sudanophilia is maintained by the presumptive endoderm of the stem, whereas the hydranth anlage shows an increase to the + 4 level characteristic of the endoderm of the adult hydranth.

Segments which failed to reconstitute even after four days of culturing provide evidence that the disintegration of the endodermal ridges may be an important link in the reconstitutive process. With the exception of their endodermal ridges, which remain intact in every instance, these non-reconstituted segments are similar to the zero point segments. The sudanophilia of their ridges is + 2; that of their circumferential endoderm is 0.

Aberrant pigment extracts

Absorption spectra of all the previously described red pigment extracts (Experiments 1, 2, and 6) contained a single absorption maximum between 460–470 mμ. However, a straw-yellow solution with a single absorption maximum between 402–410 mμ, was obtained when extracts were prepared of the reconstituting segments in three other experiments (Experiments 3, 4, and 5). The procedures followed in these experiments were identical with the following important exceptions. 1) The segments of Experiment 3 were dehydrated at 110° C. overnight for dry weight determinations before extraction. 2) The colonies used for Experiment 4 were freed of algae by dipping them into a solution of 5% $CuSO_4$ in sea water immediately before segments were cut from the stems. 3) The colonies used for Experiment 5 were immersed in 5% $CuSO_4$ 10 days before segments were amputated. During the 10-day interval between treatment and amputation, the colonies were kept in filtered sea water at 12 to 15° C.; the sea water was changed every one or two days. The hydranths autotomized after the first day; reconstitution followed in the ensuing two to three days.

In these three experiments, reconstitution occurred with the normal reddening of the hydranth anlagen. The yellow extracts of Experiment 3 were obtained from segments which were heated after removal from the culture bowls. Segments from the copper-treated colonies appeared red in life but yielded yellow pigment when extracted.

The pattern of changes in the concentration of the straw-yellow pigment during reconstitution of the heat- and copper-treated segments varied from one experiment to another and no interpretation of them will be attempted. However, the possible significance of the yellow pigment will be brought out in the Discussion.

Discussion

Origin of pigment. Short segments of *Tubularia* stems, which are free of red algae and isolated from extrinsic sources of pigment, develop the characteristic red-

dening of the hydranth-forming regions. A spectrophotometric assay has demonstrated that the total amount of pigment increases markedly in these reconstituting segments. These observations provide cogent evidence that the coenosarc of a regenerating segment synthesizes pigment. The synthesis, the nature of which is unknown, may occur throughout the whole segment or only in the hydranth-forming region.

If synthesis occurs in the entire segment, there must be a subsequent concentrating of pigment in the presumptive hydranth. The results of the present experiments offer no evidence on this point. Cohen (1952), however, has already presented evidence indicating that some re-allocation of pigment occurs during reconstitution. Using an accidentally discovered yellow form of *Tubularia,* Cohen fused 5-mm. segments of yellow stems with similar segments cut from the common red form. Subsequently, he found red pigment in the endoderm of the reconstituting "yellow" ends; these red particles gave the "yellow" hydranth a yellow-orange color. In addition, he noted that carbon particles injected into the gastrovascular fluid of a red segment were taken up differentially by the same endodermal cells which later constituted the red striations. From these observations, Cohen inferred that the linear arrangement of pigment in the primordia of the tentacles is due to a differential phagocytic activity of special endodermal cells.

Cohen's findings demonstrate that pigment already present in a stem segment can be redistributed during reconstitution. Presumably, this redistribution could occur as readily in the 2-mm. segments studied here as it did in Cohen's longer (5-mm.) segments. In a short segment, however, only a relatively small amount of pigment is present initially and synthesis of new pigment probably accounts for most of the reddening of the hydranth anlage. In long stems, containing relatively more pigment initially, re-allocation of the original stores could be a more important factor in the hydranth reddening. Therefore, it is tempting to suggest that the amount of pigment already available, either in extra- or intracellular locations, may influence the amount of pigment which will be synthesized. In this view, longer segments which contain more pigment when isolated should synthesize less pigment during reconstitution. This hypothesis could readily be tested experimentally by comparing the amount of pigment synthesized per unit wet weight of tissue in long and short reconstituting segments.

Morphogenetic significance of pigment. Previous workers have held several different opinions about the functional significance of the pigment in reconstituting *Tubularia* (see Cohen, 1952, for literature). The present work still does not permit a definitive statement but one of the following two alternatives is regarded as likely.

1) The consistent decline in the concentration of red pigment before the last phase of reconstitution (see Fig. 3), suggests that pigment or pigment-associated substances (protein, see Cohen, 1952) might be utilized in or during morphogenesis. Since the amount of tissue remained constant in the isolated segments, this decrease is a true decline in pigment concentration. There also cannot be any loss of pigment by extrusion as described by Stevens (1901, 1902) and Godlewski (1904), since the intraperisarcal hydranths were still enveloped by perisarc. Therefore, a mechanism for the breakdown of pigment may be associated with some morphogenetic activity.

2) On the other hand, the altered pigmentation—as well as the increased sudanophilia (see below)—may be merely a visible by-product of synthetic activities. If this is true, then the pigment is not causally related to morphogenesis.

Changes in sudanophilia. Reconstitution is also accompanied by marked changes in sudanophilia. Following amputation, the circumferential endoderm loses most of its sudanophilia. Concomitantly, the disintegrating endodermal ridges presumably liberate sudanophilic globules to the circulation (*cf.* the translucent globules of Stevens, 1901, 1902, and Hargitt, 1903). During reconstitution, the sudanophilia of the circumferential endoderm is gradually recovered until, at the intraperisarcal hydranth phase, levels of sudanophilia characteristic of the adult hydranth and stem are attained. In contrast, non-reconstituting segments undergo only one of these changes, namely, a decline in the sudanophilia of the circumferential endoderm.

The increase in sudanophilia of circumferential endoderm during reconstitution may result either from intracellular synthesis of new lipids, or from phagocytosis of globules previously released into the circulation—or from both. There is at present no evidence to permit a decision among these alternatives.

The relation of sudanophilia to morphogenesis is also obscure. Sudanophilic substances are increasing in the hydranth endoderm during the terminal phase of reconstitution, that is, at a time when total pigment concentration is declining. The significance of the association of these events in time is uncertain but further investigation is clearly indicated.

Disintegration of endodermal ridges. The breakdown of the endodermal ridges in reconstituting segments has been described frequently (Morgan, 1901; Stevens, 1901, 1902; Cohen, 1952). The present investigation has both confirmed these previous observations and revealed that disintegration of the ridges does not occur in segments which fail to reconstitute. These facts suggest that the breakdown of the endodermal ridges may be an important link in the reconstitutive process.

Yellow pigment extracts. The straw-yellow extracts obtained following heat or copper treatment could be due to one of the following: 1) the unmasking of a naturally occurring yellow pigment, or 2) the production of an isomeric or otherwise chemically altered form of the red pigment. There are no data which directly confirm or deny the first possibility, but evidence favoring the second has been reported by Willstaedt (1934). This investigator found that the carotenoid, astacene (presumably the red pigment in *Tubularia;* Lönnberg and Hellström, 1931 and Cohen, 1952), can be reduced by zinc dust in acetic acid-pyridine solution to give a light yellow derivative. Cohen's (1952) discovery that naturally occurring colonies of *Tubularia* may contain individuals ranging in color from red to yellow may also indicate that one type of pigment can be converted into the other.

Cohen proposes that the naturally-yellow *Tubularia* is a biochemical mutant of the red form. If this is true, and if the natural and experimentally produced yellow pigment are identical, then the heat and copper treatments have produced a phenocopy. A spectrophotometric analysis could readily decide whether or not the two yellow pigments are the same.

Summary

1. The origin of the red pigment in the hydranth-forming region of reconstituting stem segments of *Tubularia* has been investigated histologically and spectrophotometrically.

2. Short (1.5 to 2 mm.), perisarc-enclosed segments, isolated from extrinsic sources of pigment (*e.g.*, ingested materials or other non-regenerating regions of the stem), reconstitute with normal reddening.

3. Histological observations have failed to demonstrate the presence of red algae within the coenosarc of *Tubularia*. Therefore, red algae do not play a critical role in the increased coloration.

4. A spectrophotometric assay of the pigment concentration in short segments at successive intervals in the reconstitutive process has provided evidence that the total amount of pigment increases during regeneration. It is concluded that the coenosarc of regenerating segments synthesizes new pigment. This finding is of interest because the pigment is presumably a carotenoid and because no conclusive evidence has previously been presented that animals synthesize carotenoids.

5. Microscopically, pigment is found in two forms: 1) large, irregular and birefringent granules sparsely distributed in the gastrovascular fluid and in certain endodermal cells, and 2) minute anisotropic crystals occurring in large numbers in the endoderm. The reddening of the hydranth anlagen is presumably due to an increase in the number of pigment crystals.

6. During reconstitution, sudanophilic substances increase markedly in the presumptive hydranth endoderm. As the distal coenosarc endoderm transforms into hydranth endoderm, it develops the characteristic adult level of sudanophilia.

7. The possible morphogenetic roles of pigment and sudanophilic materials during reconstitution are discussed. While there is no certain relation of these substances to the morphogenetic process, further analyses are suggested.

8. The breakdown of the endodermal ridges in normally reconstituting segments, and the lack of endodermal ridge breakdown in non-reconstituting segments, indicate a possible role of these structures in the regenerative process.

9. A straw-yellow pigment, chemically different from the characteristic red pigment, is obtained when reconstituting segments are treated with heat or copper sulfate. This yellow pigment may be similar to that occurring occasionally in natural populations of *Tubularia*.

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EXPLANT SYSTEMS AND THE REACTIONS OF GASTRULATING AMPHIBIANS TO METABOLIC POISONS

JOHN R. GREGG AND NORMA ORNSTEIN

Department of Zoology, Columbia University, New York 27, New York

Gastrulating amphibian embryos are complex systems of moving parts whose displacements relative to each other may be slowed down, or even stopped, in environments differing in but few respects from normal ones—differing, say, only in the presence of minute traces of salts that act as metabolic poisons or in being saturated with nitrogen instead of with air. But our information about such environmentally impaired systems is descriptively incomplete, even from the standpoint of gross morphology. The parts of gastrulating embryos belong to several different and embryologically interesting morphological types—to different germ layers, for example—and their relative shifts of position are of more than one morphological kind (for a recent classification see Nelsen, 1953). Yet, in general, direct observation of inhibited embryos yields only the information that they lag, morphogenetically speaking, behind embryos developing in more favorable circumstances; and it is difficult, if not impossible, to say whether a given inhibitor has affected movements of all or only of some of the germ layers, or to decide which among the distinguishable kinds of movements have been altered.

Clearly, some more adequate method of observation is needed. It is the chief aim of this paper to suggest that such a method is provided by Holtfreter's recent (1944) directions for constructing explant systems in which there occur in relative isolation more or less homologous counterparts of morphogenetic movements known since Vogt's classical work to characterize gastrulating whole embryos. Holtfreter has shown, for example, that the two parts of an explant system consisting of an endodermal ball surmounted by a piece of coated ventral ectoderm will exhibit a behavior reminiscent of epiboly. The ectoderm spreads glacier-like down over the endoderm on all sides, much as in normally gastrulating embryos. With the help of such models, observed developing in inhibitory environments, it should be possible to specify the morphological sites of action of inhibitors in as much detail as ingenuity in constructing adequate explant systems permits. A second aim of this paper is to cite the results of some exploratory experiments designed to show that information of the expected kind is in fact forthcoming. Yet we wish to stress that our relatively crude study must be refined and extended to obtain results of any genuine importance; and for this reason our discussion will be less extensive than one appropriate to more detailed experimental analysis.

Methods

General

Suitable explants were obtained by dissecting *Rana pipiens* embryos at Stage 10 (early dorsal lip; Shumway, 1940) in full-strength Holtfreter's solution with bi-

carbonate at pH 8.0 (hereafter called "standard solution"). Immediately afterwards they were assembled into explant systems in inhibitor solutions (experimental systems) and in standard solution (control systems). At the same time, whole embryos were placed under experimental and control conditions exactly similar to those just mentioned. Explant systems and whole embryos were allowed to develop for 16

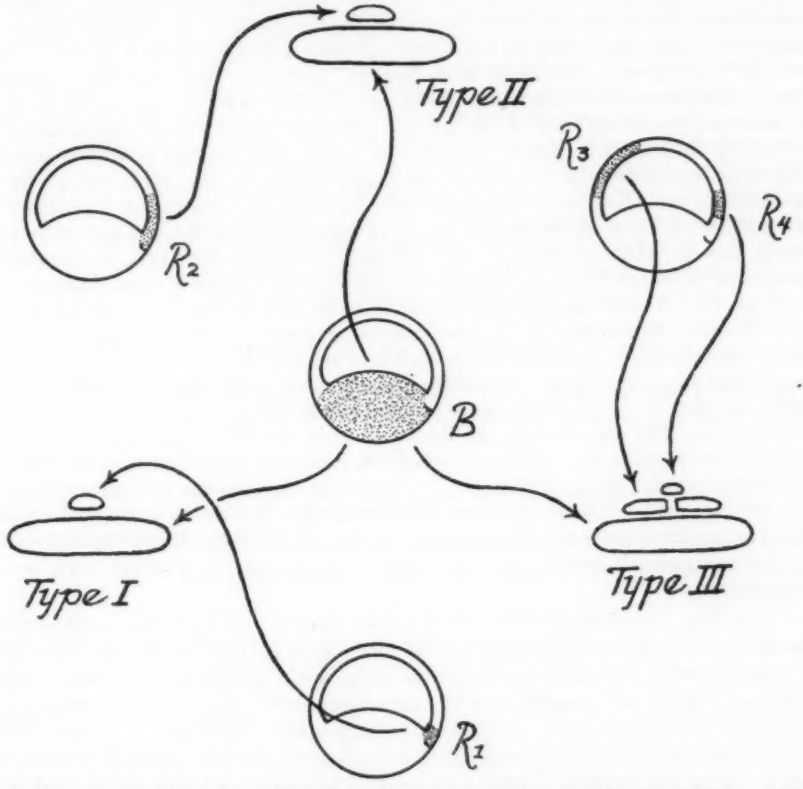

FIGURE 1. Assembly of explant systems. Whole gastrulae shown with dorsal sides to right. B: stippled area represents site of origin of endodermal bases. R_1: site of origin of Type I riders (presumptive head endoderm). R_2: site of origin of Type II riders (presumptive head endoderm and presumptive notochord). R_3 and R_4: sites of origin of Type III rider-components (presumptive epidermis and presumptive notochord, respectively). At eight, twelve and four o'clock, assembled explant systems are shown with coated surfaces of endodermal bases down, coated surfaces of riders up.

to 44 hours at room temperature (20 to 25° C.) except in experiments on anaerobiosis at 12 to 14° C. In the latter case, both experimental and control media were standard solution made 0.02 *M* in bicarbonate. Anaerobic environments were obtained and tested exactly as described by Gregg and Ornstein (1952).

Explant systems

We have worked thus far with three different sorts of explant systems (all described by Holtfreter). Each system consists of an endodermal *base* (the entire yolk mass of a Stage 10 gastrula) and a *rider* of one kind or another (see Fig. 1).

Type I. In a system belonging to this type the rider is a small piece of coated presumptive head endoderm removed from just over the dorsal lip of a Stage 10 gastrula and set on the uncoated blastocoel floor surface of an endodermal base. During the hours succeeding their assembly, Type I systems exhibit a behavior that we call "endodermal embedding in endoderm" (briefly, "Em(en, en)"); for the riders embed themselves slowly into the bases until their surface coats are flush with the uncoated upper surfaces of the bases. (We have not observed the pitting described by Holtfreter, probably because our riders were always relatively large.) Holtfreter has studied these systems histologically (see his Fig. 16), and we have his authority for it that the riders invade their bases by the formation of "bottle cells" in a manner wholly reminiscent of a similar process presaging the first appearance of dorsal lips in whole gastrulae. We follow Holtfreter, therefore, in regarding Em(en, en) as a model of this normally-occurring morphogenetic movement.

Type II. A rider belonging to a system of the second type is a surface-coated piece of Stage 10 gastrula-tissue containing some presumptive head endoderm and also some mesoderm (presumptive notochord) set initially upon the uncoated surface of an endodermal base. Following the assembly of a Type II system, the endodermal portion of its rider executes the movement Em(en, en). The mesodermal portion of its rider may or may not invade its base, but will always elongate in the original meridional axis. We have called this behavior "mesodermal stretching on endoderm," or, for short, "St(m, en)." Holtfreter regards this movement (see his Fig. 14) as a model of convergent stretching, which is exhibited by presumptive notochord immediately prior to its invagination, or as a model of the elongation undergone by just-invaginated presumptive notochord. Again, we adopt Holtfreter's interpretation.

Type III. A system of this sort has the usual endodermal base with a rider mounted on its uncoated surface; but the rider itself is compounded of a flat sheet of coated ventral ectoderm (presumptive belly skin) pierced in the center with a hole on which is set a small piece of coated mesoderm (presumptive notochord). Type III systems, therefore, are like those illustrated in Holtfreter's Figure 22 except for the substitution of presumptive notochord for lateral marginal-zone mesoderm. Type III systems exhibit two sorts of behavior. (1) The ectodermal portion of the rider spreads out over the endodermal base in a manner that we interpret, again following Holtfreter, as a model of the epibolic extension of ectoderm in intact gastrulating embryos. We call this behavior "ectodermal spreading on endoderm," or "Sp(ec, en)." (2) The mesodermal part of the rider fuses smoothly with the ectodermal part, in an activity we have called "mesodermal fusion with ectoderm on endoderm," or "Fu(m, ec, en)." This particular behavior does not clearly model any normally-occurring gastrular event. We had hoped to be able to duplicate the events sketched in Holtfreter's Figure 22, which can be reasonably interpreted as modelling the formation of a rudimentary archenteron by mesodermal tissue, but we were never able clearly to obtain this result. Nevertheless, we report

experiments with Fu(m, ec, en) because it is differentially affected (relative to other model movements) in some inhibitory environments.

Total carbohydrate

To obtain information about carbohydrate utilization by gastrula explants under anaerobiosis, dorsal and ventral explants (usually about 8 each) from Stage 10 gastrulae were divided into lateral halves. Right halves (DR, VR) were made anaerobic, left halves (DL, VL) *from the same gastrulae* were kept as controls. After 20.5–21.5 hours at 12–14° C., in standard solution made 0.02 *M* in bicarbonate, explants were homogenized in a known volume of standard solution, aliquots of brei were hydrolyzed for three hours in ca. 2 per cent HCl, proteins were precipitated by treatment with copper and acid tungstate, and reducing sugars were estimated by the method of Kirk (1950) and Stern and Kirk (1949). Other aliquots of brei were taken for dry weight measurements with a quartz helix microbalance. Under these conditions we were able to recover 98 per cent of known amounts of glucose added to brei (in the range 1–12 μg.) with a reproducibility of about 2 per cent.

The explant-types are those described by Ornstein and Gregg (1952); a fuller description of the method of weighing is to be found in this publication also.

Development of Whole Embryos in Inhibitory Environments

The developmental behavior of gastrulae placed at Stage 10 under inhibitory conditions is summarized in Table I. Generally speaking, we have chosen to work

TABLE I

Development of amphibian gastrulae exposed to inhibitory environments at Stage 10. Temperatures, 20–25° C.

Inhibitor	Conc'n. molar	pH	Exposure, hours	Developmental stage at end of exposure		No. clutches tested
				Expts.	Controls*	
Azide	0.005	8.2	16–20	10^+–11	12–14	2
Malonate	0.045	8.3	22	11^+†	14	1
	0.040	8.0	17–18	11–11^+	12^+–13§	2
NaCl‡	0.045	8	18–24	11^+†	13–15	3
	0.022	8	18–22	12–14	13–14	2
p-chloromercuribenzoic acid	5×10^{-4}	8.2	16–20	11–11^+	12^+–15	4
2,4-dinitrophenol	1.1×10^{-4}	8.0–8.2	17–21	10^+	13–15	5
Sodium barbital	0.05	9.4	20	11^+–12^-	14	1
Urethane	0.1	8	44	10^+	18	1
	0.05	8	44	14^+	18**	1
95 N_2:5 CO_2	Saturated††	7.2–7.4 (calc.)	16–18	10^+–11^+	12^+–14	3

* In standard solution at pH 8.0.
† Head-fold induction in these gastrulae, in spite of incomplete invagination.
‡ Not regarded as a metabolic poison; see text section on malonate.
§ Another (atypical) control set developed only to Stage 11^+.
** Some persistent fairly large yolk plugs.
†† In standard solution made 0.02 *M* in bicarbonate.

with inhibitors in concentrations that slow or stop gastrulation-movements at the pH's indicated, and yet which result in minimum damage to exposed embryos—damage in the sense of visible cytolysis, surface-coat corrosion, and so on. Where some visible injury has been unavoidable, it will be mentioned in an appropriate place.

To reiterate a point made earlier: with some exceptions, inhibited embryos differ visibly only in minor respects from normal ones, the chief difference being that they develop more *slowly* than normal ones. In some instances, of course, developmental processes are brought to a standstill, or nearly so.

Development of Explant Systems in Control and in Inhibitory Environments

The data will be presented piece-meal as a sequence of formulae interspersed with commentary. For this purpose a simple and brief notation has been adopted.

If R is a type of response exhibited by successful control systems, C is the set of environmental conditions regarded as relevant to R, P is the set of positive results (the movement definitely occurred) obtained in the experiments, N is the set of negative results (the movement definitely failed to occur), I is the set of indeterminate results (undecidable except by vote), and m, n, p and q are integers, then the expression:

$$\mathrm{R:C;\ (}m\mathrm{)\ ;\ }n\mathrm{P;\ }p\mathrm{N;\ }q\mathrm{I}$$

will be read as asserting that out of m attempts to obtain responses of type R under environmental conditions of Type C, n were successful, p were unsuccessful and q were indeterminate. A fictional instance may help to make this idiom perfectly clear. The formula:

$$\mathrm{Em(en,\ en):\ NaN_3,\ 0.01\ }M\mathrm{;\ (7)\ ;\ 4P;\ 1N;\ 2I}$$

tells us that out of 7 attempts to obtain endodermal embedding in endoderm in 0.01 M sodium azide, 4 were successful, 1 was unsuccessful, and 2 were indeterminate.

Parenthesized numerals preceding formulae indicate merely their order of presentation.

Control environments

Because of their almost uniformly unambiguous success, data on all the control experiments are presented together, classified according to the kind of movement studied.

(1) Em(en, en): standard solution; (24); 24P; 0N; 0I
(2) St(m, en): standard solution; (30); 29P; 0N; 1I
(3) Sp(ec, en): standard solution; (22); 22P; 0N; 0I
(4) Fu(m, ec, en): standard solution; (22); 22P; 0N; 0I

The behavior of these explant systems under control conditions (standard solution in all experiments except those on anaerobiosis; in these, standard solution made 0.02 M in bicarbonate served both as experimental and as control medium) is astonishingly reliable and reproducible. Aside from preliminary trials during which we were learning how to assemble the systems, and whose results are not reported

here, we have rarely had to discard an experiment because of unsatisfactory behavior on the part of control systems. No uncontrolled experiments are reported.

Sodium azide (NaN_3)

The results of experiments with azide:

(5) Em(en, en) : NaN_3 0.005 *M*; (4); 4P; 0N; 0I
(6) St(m, en) : NaN_3, 0.005 *M*; (6); 0P; 6N; 0I
(7) Sp(ec, en) : NaN_3, 0.005 *M*; (5); 0P; 5N; 0I
(8) Fu(m, ec, en) : NaN_3, 0.005 *M*; (5); 5P; 0N; 0I

indicate that explant systems immersed in weak solutions of it fail to exhibit movements of the types St(m, en) and Sp(ec, en), but are perfectly normal in respect to movements of the kinds Em(m, en) and Fu(m, ec, en). Therefore, insofar as our models do not simply parody the *in situ* movements of gastrulating embryonic parts, we may reasonably suppose that whole gastrulae are blocked by immersion in azide solutions (see Table I) not because such environments are inimical to bottle-cell formation but partly, at least, because convergent stretching and epibolic spreading are somehow interfered with. Furthermore, we can say (although the morphogenetic import of this is not quite clear) that azide does not abolish whatever conditions are necessary for mesoderm and ectoderm to fuse normally.

It should be noted that in azide the ectodermal parts of riders in Type III systems fail to adhere well to the endodermal bases, and this suggests that some cell-surface feature is altered that under more favorable circumstances allows endoderm as a suitable substrate to which ectoderm can stick while spreading.

Sodium malonate (*Mal*)

Our experiments with sodium malonate have yielded results that in some respects are confusing. These are reported, nevertheless, in caution against accepting claims for specific inhibitor effects obtainable only when the inhibitors are employed in relatively high concentrations.

Reference to Table I will show that whole gastrulae are developmentally slowed in 0.045 *M* and also in 0.040 *M* malonate solutions. From a biochemical point of view, this result is rendered less interesting by the discovery, also cited in Table I, that the same effect is obtainable with 0.045 *M* NaCl. This is pointedly suggestive of a purely osmotic effect on the part of malonate. Perhaps the osmotic pressure of the blastocoel is elevated by the penetration of malonate to such an extent that the resulting increase of blastocoel hydrostatic pressure makes complete invagination impossible, or perhaps some osmotic damage to gastrular cells is incurred.

Whatever the exact situation, it is not greatly illuminated by the behavior of explant systems in malonate solution. In 0.045 *M* malonate no movements belonging to any of the four types are seen:

(9) Em(en, en) : Mal, 0.045 *M*; (2); 0P; 2N; 0I
(10) St(m, en) : Mal, 0.045 *M*; (2); 0P; 2N; 0I
(11) Sp(ec, en) : Mal, 0.045 *M*; (2); 0P; 2N; 0I
(12) Fu(m, ec, en) : Mal, 0.045 *M*; (2); 0P; 2N; 0I

Yet in 0.040 *M* malonate (in which whole gastrulae develop exactly as they do in 0.045 *M* malonate) some of the movements, notably St(m, en) and Fu(m, ec, en), occur with a relatively high frequency of success:

(13) Em(en, en) : Mal, 0.040 *M* ; (7) ; 3P ; 2N ; 2I
(14) St(m, en) : Mal, 0.040 *M* ; (8) ; 6P ; 2N ; 0I
(15) Sp(ec, en) : Mal, 0.040 *M* ; (8) ; 3P ; 5N ; 0I
(16) Fu(m, ec, en) : Mal, 0.040 *M* ; (8) ; 6P ; 2N ; 0I

The asymmetry of all these results is increased by the behavior of explant systems in 0.045 *M* NaCl, which, unexpectedly, behave quite normally except in respect to Sp(ec, en) :

(17) Em(en, en) : NaCl, 0.045 *M* ; (2) ; 2P ; 0N ; 0I
(18) St(m, en) : NaCl, 0.045 *M* ; (2) ; 2P ; 0N ; 0I
(19) Sp(ec, en) : NaCl, 0.045 *M* ; (2) ; 0P ; 1N ; 1I
(20) Fu(m, ec, en) : NaCl, 0.045 *M* ; (2) ; 2P ; 0N ; 0I

We have no results with explant systems in 0.04 *M* NaCl; but in 0.22 *M* NaCl they are apparently perfectly normal, and whole gastrulae are affected but little, if at all (Table I).

Incidentally, the warning with which this section opened applies to our own claim (Ornstein and Gregg, 1952) that explant respiration is reduced in 0.04 *M* sodium malonate. We stand by this assertion, but the experiments yielding it would now seem to need revaluating.

Para-chloromercuribenzoic acid (HgB)

This powerful -SH inhibitor is definitely capable of blocking Sp(ec, en) and probably St(m, en) ; its effect on Fu(m, ec, en) is undetermined by our experiments; and it has no effect, in the concentrations employed, upon Em(en, en) :

(21) Em(en, en) : HgB, 5×10^{-6} *M* ; (4) ; 4P ; 0N ; 0I
(22) St(m, en) : HgB, 5×10^{-6} *M* ; (4) ; 0P ; 2N ; 2I
(23) Sp(ec, en) : HgB, 5×10^{-6} *M* ; (4) ; 0P ; 4N ; 0I
(24) Fu(m, ec, en) : HgB, 5×10^{-6} *M* ; (4) ; 0P ; 0N ; 4I

It should be remarked that p-chloromercuribenzoic acid tends to thin out or corrode the surface coats of explants [1] or of whole embryos. Under the present experimental conditions there were always some loose cells lying at the outer surfaces of the explant systems and impeding observation. This accounts for our inability to decide whether Fu(m, ec, en) occurs in solutions of this inhibitor (24) and for the indeterminacy of some of the results mentioned in (22). It seems to account also for the negative results reported in (23) ; for the ectodermal portions of the riders of Type III systems, denuded by p-chloromercuribenzoate of their surface coats, behaved as though they were endodermal pieces and embedded themselves in the endodermal bases instead of spreading over them. These observations confirm Holtfreter's report (1944) that uncoated explants of almost any sort will behave in this manner, relative to an endodermal substratum.

[1] We wish to correct an inadvertent error of omission in Ornstein and Gregg, 1952: in that publication we failed to mention that explants in 10^{-4}–10^{-5} *M* p-chloromercuribenzoic acid become heaps of loose cells during their two-hour stay in the respirometers. Presumably this occurs because their surface coats are disrupted, for their cells are otherwise not visibly affected. This change is unaccompanied by alteration of respiratory rate, *i.e.*, the latter stays constant during the period of measurement. For weighing, the dispersed cells were removed with a braking pipette.

It is interesting to note that Em(en, en) proceeds in a perfectly normal way. Evidently embryos do not fail to complete gastrulation because bottle-cell formation is retarded by this -SH inhibitor.

2,4-Dinitrophenol (DNP)

Gastrulae immersed at Stage 10 in 1.1×10^{-4} *M* DNP at once cease developing (Table I). Yet under these conditions explant systems behave very much as they do in standard solution:

(25) Em(en, en) : DNP, 1.1×10^{-4} *M*; (5) ; 5P ; 0N ; 0I
(26) St((m, en) : DNP, 1.1×10^{-4} *M*; (8) ; 6P ; 0N ; 2I
(27) Sp(ec, en) : DNP, 1.1×10^{-4} *M*; (2) ; 2P ; 0N ; 0I
(28) Fu(m, ec, en) : DNP, 1.1×10^{-4} *M*; (2) ; 2P ; 0N ; 0I

There were two peculiarities: in DNP, Sp(ec, en) was somewhat less extensive than usual; and there was some notochordal self-differentiation in Type III systems, atypical in systems of this type.

It appears, therefore, that failure of some morphogenetic movement not modelled by our explant systems blocks whole gastrulae exposed to 1.1×10^{-4} *M* DNP. It should not be supposed, however, that Em(en, en) and the others *cannot* be blocked by DNP; for explant systems developing in 1.6×10^{-4} *M* DNP do not exhibit *any* of these movements.

Sodium barbital (Barb)

In solutions made 0.01–0.02 *M* in sodium barbital, embryos will gastrulate at a normal rate; at these concentrations explant systems, also, behave just as they do in standard solution. But in 0.05 *M* sodium barbital, gastrulating embryos are greatly retarded (Table I), and explant systems are affected also. Only Fu(m, ec, en) continues to occur, and even this activity is less well-marked than usual:

(29) Em(m, en) : Barb, 0.05 *M*; (1) ; 0P ; 1N ; 0I
(30) St(m, en) : Barb, 0.05 *M*; (1) ; 0P ; 1N ; 0I
(31) Sp(ec, en) : Barb, 0.05 *M*; (1) ; 0P ; 1N ; 0I
(32) Fu(m, ec, en) : Barb, 0.05 *M*; (1) ; 1P ; 0N ; 0I

No peculiarities of explant systems in sodium barbital were observed, other than that the endodermal bases were less turgid and more limp than usual.

Urethane (Ur)

Whole gastrulae at Stage 10 will develop with occasional abnormalities following immersion in 0.05 *M* urethane, while in 0.1 *M* urethane their development is curtailed almost at once (Table I). But, in environments of both sorts, St(m, en) is selectively inhibited. Data for 0.05 and 0.1 *M* urethane are lumped together, since there are no results on explant systems that differentiate them:

(33) Em(en, en) : Ur, 0.05, 0.1 *M*; (4) ; 3P ; 0N ; 1I
(34) St(m, en) : Ur, 0.05, 0.1 *M*; (4) ; 0P ; 4N ; 0I
(35) Sp(ec, en) : Ur, 0.05, 0.1 *M*; (4) ; 4P ; 0N ; 0I
(36) Fu(m, ec, en) : Ur, 0.05, 0.1 *M*; (4) ; 4P ; 0N ; 0I

Thus, while sodium barbital seems to act according to the classic picture of narcotics as general cell-poisons, urethane does not; and since we are regarding St(m, en) as more than a mere caricature of convergent stretching, it might seem tempting to suppose (from (34) and Table I) that whole gastrulae fail to develop in 0.1 *M* urethane because they cannot therein exhibit convergent stretching. But this would be a highly dubious interpretation, for they will often finish gastrulating in 0.05 *M* urethane, in which St(m, en) is also suppressed.

Anaerobiosis, 95 N_2: 5 CO_2 (An)

Gastrulae made anaerobic at Stage 10 are sometimes prevented thereby from developing much further (Table I); but under similar conditions there will occur all four of the surrogate movements that we have been discussing:

(37) Em(en, en) : An; (3) ; 3P ; 0N ; 0I
(38) St(m, en) : An; (2) ; 2P ; 0N ; 0I
(39) Sp(ec, en) : An; (2) ; 2P ; 0N ; 0I
(40) Fu(m, ec, en) : An; (2) ; 2P ; 0N ; 0I

Yet Sp(ec, en) is noticeably less extensive in nitrogen than in air, and it is therefore possible that gastrulae are retarded in anaerobic environments at least partly because in such circumstances epibolic spreading is curtailed.

Total Carbohydrate in Anaerobic Embryos and Explants

We turn now to giving an account of some experiments that differ somewhat in technique from the foregoing, but that are relevant to the interpretation of the latter and to the whole question of gastrula metabolism.

The work of Heatley (1935), and especially of Jaeger (1945), has convinced all but the most critical that, in the process of invaginating, gastrular tissues lose about 30 per cent of their glycogen. When Jaeger showed that gastrula explants are not self-differentiating in this respect, she suggested among other things (possibly in response to a hint by Barth, 1942) that invaginated tissues are brought normally into regions under low oxygen-tension, and that mediation by a Pasteur effect might therefore account for their accelerated glycolytic activity. As she pointed out, this hypothesis is susceptible to test, and she announced that experiments to this end were in progress. These were never completed, but preliminary trials failed to support her suggestion (personal communication to the authors). Because of the general interest attached to this question, we have undertaken to extend her preliminary experiments and have confirmed them completely.

Table II

Effect of anaerobiosis upon total carbohydrate content of gastrula explants. Numerals following the ± sign indicate standard deviations. P-values calculated by t-test.

	μg. total carbohydrate/μg. dry wt. after 24 hours at 12–14° C.			
	Anaerobic	Control	P	No. expts.
Dorsal explants	DR: 0.22±0.02	DL: 0.20±0.02	>0.05	3
Ventral explants	VR: 0.21±0.03	VL: 0.20±0.04	>0.05	3

Our results are presented in Table II. They show clearly that, relative to aerobic controls, no significant change occurs in the carbohydrate content of explants, even after 20–21 hours anaerobiosis at 12°–14° C., under which conditions control gastrulae have reached early Stage 12. But if the hypothesis being tested were correct, these systems should have lost about 30 per cent of their glycogen: a carbohydrate change that would have been easily detected (see METHODS). Consequently, the hypothesis is not supported by the evidence, and the reasons for the disappearance of large amounts of glycogen from invaginating gastrular tissues remain quite obscure.

The moral to be drawn is this: the interpretation of explant behavior, metabolic or otherwise, may be complicated by inexplicable responses to isolation.

Summary and General Comments

We have proposed a way to analyze the developmental failure of embryos exposed to unfavorable environmental circumstances; for instance, to anaerobiosis or to metabolic poisons. Our method is based upon the supposition, not clearly warranted by direct observation of delinquent but intact embryos, that under such conditions the component morphogenetic events of embryonic development are selectively retarded in a manner indirectly identifiable by reference to the behavior under similar conditions of explant systems whose overt movements in favorable environments constitute *models* of normal morphogenetic displacements.

In a crude preliminary demonstration of the utility of the method, we have studied three types of explant systems described by Holtfreter. Systems of these types exhibit severally three sorts of movements—called "Em(en, en)" (endodermal embedding in endoderm), "St(m, en)" (mesodermal stretching in endoderm) and "Sp(ec, en)" (ectodermal spreading on endoderm)—that may be regarded with some plausibility as respectively modelling bottle-cell formation, convergent stretching and epibolic expansion. In addition, one such system exhibits a fourth kind of movement—we have called it "Fu(m, ec, en)" (mesodermal fusion with ectoderm on endoderm)—whose instances do not clearly mimic any sort of morphogenetic event occurring in intact gastrulae.

The method seems to work. To the extent that our models are adequate, we have been able to demonstrate some differential effects upon gastrular movements of various inhibitory environmental agents. Our findings may be summarized in the following way:

(1) Surprisingly, 2,4-dinitrophenol (a respiratory stimulant) and 95 N_2: 5 CO_2 act alike in depressing Sp(ec, en) but not Em(en, en), St(m, en) or Fu(m, en, en). As a tentative guide to discovery, therefore, we might assume that gastrulae fail in dinitrophenol, and are slowed down in N_2: CO_2, partly because epiboly (but neither bottle-cell formation nor convergent stretching) is suppressed in these circumstances.

(2) Azide, the -SH reagent p-chloromercuribenzoic acid and urethane are inhibitory to both St(m, en) and Sp(ec, en). They can, therefore, be assumed to retard gastrulation movements at least in part because in their presence convergent stretching and epiboly either cannot occur or else are retarded (as Sp(ec, en) by urethane). None of the three inhibitors affects Em(en, en) (and, hence, bottle-

cell formation). Fu(m, en, en) normally occurs in azide and in urethane. Whether it can occur in p-chloromercuribenzoic acid has not been determined by our experiments.

(3) Among the inhibitors that we have studied, sodium barbital is unique in having no observable effect upon Em(en, en), St(m, en) or Sp(ec, en), but in retarding Fu(m, ec, en) to some extent. The precise embryological interest of the last result is not clear.

(4) Sodium malonate in certain concentrations (0.04 *M*) suppresses all four activities of explant systems, but most extensively Em(en, en) and Sp(ec, en). Whatever interest this result may have is somewhat lessened by the finding that NaCl in similar concentrations is equally effective.

From an embryological point of view our results are too sketchy, and from the standpoint of biochemistry the exact modes of inhibitor-action are too little understood, to warrant further postulates linking up metabolic events in gastrulating embryos with morphological ones. And, clearly, the method of analysis we have proposed is limited. Before really fruitful information can be obtained with it (fruitful in the sense of suggesting powerful explanatory hypotheses) a careful classification of morphogenetic movements will have to be made, suited to the demands of this type of experiment. Ingenuity will have to be exercised to design and construct refined and more adequate explant models—with due attention paid to unexplained metabolic vagaries of isolated embryonic tissues, as Jaeger's work with glycogen has warned us. And chemically more specific inhibitors whose modes of action are well-understood will have to be employed (metabolite analogs offer some attractive possibilities here). Nevertheless, in theory, there seems to be no reason why metabolic analyses of developmental events cannot be profitably undertaken in the way proposed.

The authors wish to acknowledge more specifically than in the text the stimulus of Professor Holtfreter's careful and beautiful work with explant systems.

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PARTICLE FILTRATION IN SOME ASCIDIANS AND LAMELLIBRANCHS [1]

C. BARKER JØRGENSEN AND EDWARD D. GOLDBERG

Laboratory of Zoophysiology, University of Copenhagen, and Scripps Institution of Occanography, University of California, La Jolla, California

Suspension feeders in the sea obtain their food by filtering finely particulate organic matter from the surrounding water. This suspended organic material ranges in size from macroscopic particles down to colloidal dimensions. It is of importance, therefore, to know how effectively filter feeders retain particles of different sizes. Previous investigations on the dependency of particle retention in lamellibranch gill of particle size have given varying results. Galtsoff (1928) reported that the oyster *Crassostrea virginica* effectively filtered diatoms and dinoflagellates, whereas 70–90% of *Bacterium coli* escaped the gill filter. According to Loosanoff and Engle (1947), however, even 60 μ cells of the flagellate *Euglena* could easily pass through the gills of *Crassostrea virginica*, only 15–80% being strained from the water. ZoBell and Landon (1937) stated that the gills of *Mytilus californianus* retain bacteria very efficiently. In *Mytilus edulis* Jørgensen (1949a) found an almost complete retention of particles a few micra in diameter. In order to determine whether a difference exists in the straining efficiency between the oyster and the mussel gills, further investigations were made on *Crassostrea virginica* and *Mytilus edulis*.

In a previous paper (Jørgensen, 1949b) it was stated that the ascidian *Ciona intestinalis* strained particles even more effectively than did *Mytilus*. In this paper are reported attempts to determine the efficiency with which different proteins are adsorbed by the feeding mucus of *Ciona*.

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TECHNIQUE

The relative retention of particles of various sizes was determined by comparing the rates at which the particles were removed from suspensions. The rate of removal is a function of both the amount of water transported through the animal per unit time and the retentive efficiency. However, it was found that when the animals were adapted to the experimental conditions and were not disturbed, the rate of water transport was rather constant (see Fox, Sverdrup and Cunningham, 1937). In some experiments two types of particles were present in suspension simultaneously. In such experiments, of course, differences in the rates of removal were directly indicative of differences in retention efficiency.

[1] Contribution from the Scripps Institution of Oceanography, New Series No. 650.

Suspensions were composed of (1) diatoms (*Asterionella japonica* which form colonies about 200 μ in diameter); (2) colloidal graphite (Aquadag A, obtainable from the Acheson Colloids Corporation, Port Huron, Michigan, U. S. A.); (3) blood hemocyanins (from a crab *Loxorhynchus grandis* and from a gastropod, *Haliotis* sp.); and (4) hemoglobin (from man and a fish *Amphisticus argenteus*).

The colloidal graphite forms stable suspensions in distilled water. Suspensions in sea water were prepared from stock suspensions in distilled water. In sea water, the graphite particles coagulate slowly, and in one or a few days all graphite is deposited as macroscopic particles. The coagulation process proceeds very slowly, however, during several hours following the preparation of the suspension. Measurements have been made of particle sizes in freshly prepared and two hour-old

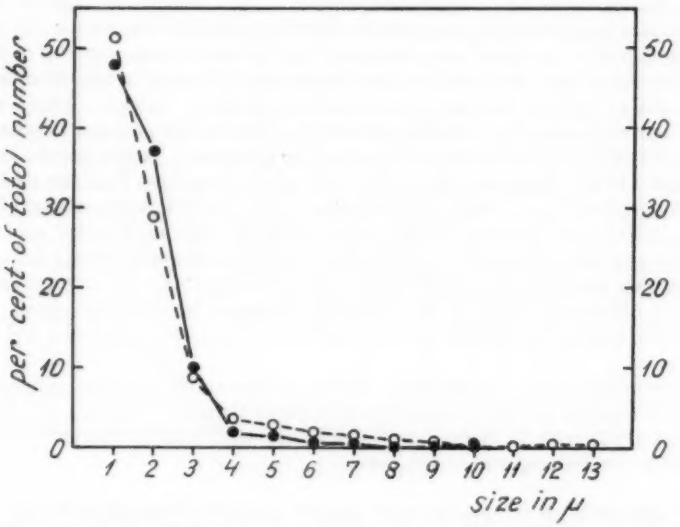

FIGURE 1. Size distribution of graphite particles (Aquadag). ○---○, sampled immediately after preparation of the suspension; 505 particles measured. ●—● sampled after 2 hours' agitation with magnetic stirrer; 552 particles measured.

suspensions (Fig. 1). In these examples, it is seen that no, or only small, changes occurred in particle size. By far the greater number were 1–2 μ in diameter. In other instances, however, it was observed that the dominating size increased to 2–3 μ during the first half hour. Thereafter practically no further coagulation might occur during the following 5 hours. The suspensions used in the experiments were never more than some 5 hours old.

The graphite concentrations were determined colorimetrically by means of a Beckman Model DU Spectrophotometer or a Klett-Summerson photoelectric colorimeter. The initial concentrations of the graphite were 0.2–0.5 mg./liter. It was found that the light absorption, expressed as a function of the concentration of graphite, followed Beer's Law. In experiments wherein the graphite was com-

pletely retained in the feeding organs, the proportion of particles removed from the water could therefore directly be determined from the corresponding decrease in light absorption. This, of course, was not strictly permissible when the graphite particles were only incompletely retained in the filters. Under such conditions large particles were removed more effectively than small particles. Since large particles absorb more light than do small particles, the numerical fraction of particles removed was consequently smaller than might correspond to the decrease in light absorption.

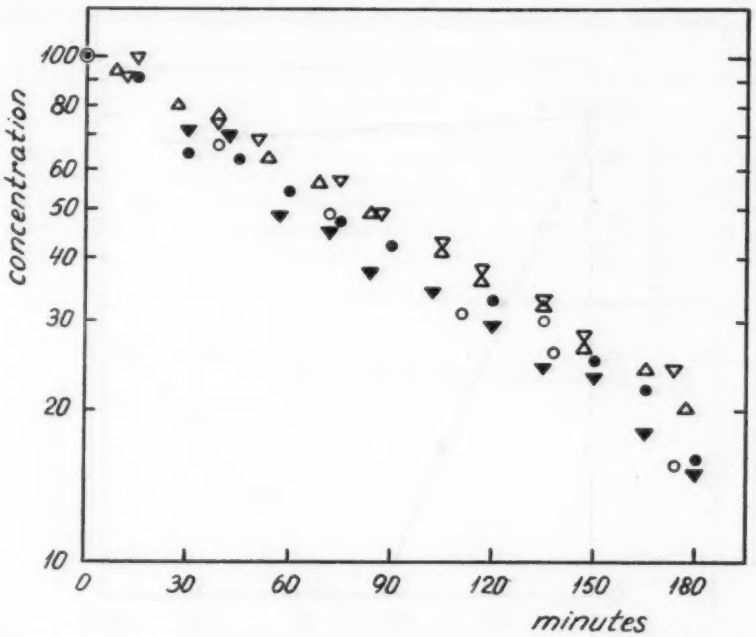

FIGURE 2. *Ciona intestinalis.* Rate of removal of diatoms (● ▼) and colloidal graphite (○ ▽ △). Abscissa: Time in minutes. Ordinate: Concentration in per cent of initial.

The diatoms which were used in the experiments had been cultivated in solutions containing radioactive phosphorus [2] as $P^{32}O_4^{---}$, and had assimilated essentially all the phosphate from the culture media. Samples of the test solution containing the diatoms were evaporated to dryness in planchets under an infrared lamp and assayed for activity under an end-window Geiger-Müller tube with a 1.4 mg./cm.^{2} window thickness. Corrections were made for the small amount of activity leaking into the water from the diatoms.

[2] The radioactive P^{32} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission. See also E. D. Goldberg, T. J. Walker and A. Whisenand (1951).

The hemocyanin and the hemoglobin were assayed spectrophotometrically in 5- or 10-cm. cells. The former suspension was dyed with Evans Blue, T 1824, and excess dye was removed by dialysis against sea water. The measurements were made at 6300 Å. Hemoglobin solutions were obtained by hemolyzing the blood which was eventually diluted to one part per 7000 parts of sea water. The measurements were made at 5700 Å.

Experiments were made on from one to six healthy specimens at a time. Prior to a given experiment, the animals were adapted to the experimental conditions for one to several days. They were kept in 1.0- or 3.5-liter glass vessels. The suspended materials were added cautiously to avoid disturbing the animals. Effective

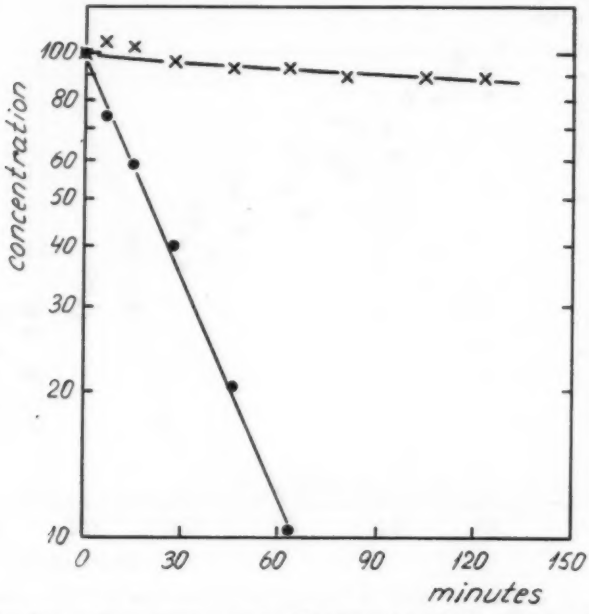

FIGURE 3. *Ciona*. Rate of removal of colloidal graphite (●) and hemoglobin (×) simultaneously present in suspension.

mixing was secured by aeration of the aquaria and aided by the pumping action of the animals. Controls were maintained without animals to ascertain whether any sedimentation or adsorption of the suspended matter occurred during the period of an experiment. In no case did this occur; hence the removal of the suspension was presumed to be due only to the filtering activity of the animals.

Usually the rate of removal of the different substances was determined by separate experiments. In the case of *Ciona intestinalis*, two suspensions, hemoglobin and graphite, were applied simultaneously. The light absorption was measured at 6500 Å and at 5700 Å. At the former wave-length the extinction is almost ex-

clusively due to the presence of graphite, whereas at the latter wave-length the hemoglobin contributes through an absorption maximum. Graphite was found to absorb equally at both wave-lengths. The specific extinction of graphite, which was computed from the readings at 6500 Å, could therefore be used as a "blank." The specific extinction of hemoglobin was obtained by subtracting the extinction of graphite from the extinction at 5700 Å.

Results

Ciona intestinalis (L.):

Ciona intestinalis specimens were taken both from Mission Bay and from the San Diego Yacht Club Harbor, San Diego, California. Single specimens or clusters of from two to six were used in the experiments.

The relative efficiency with which graphite particles were removed by *Ciona* was determined by comparing the rate of removal of graphite with that of colonies of *Asterionella japonica* which are bigger than the apertures of the branchial basket (Roule, 1884). Hence, all colonies must be filtered from the water passing the pores. Figure 2 shows some typical results of the rate of removal of graphite and the diatom colonies. In these and the following graphs, the logarithm of the

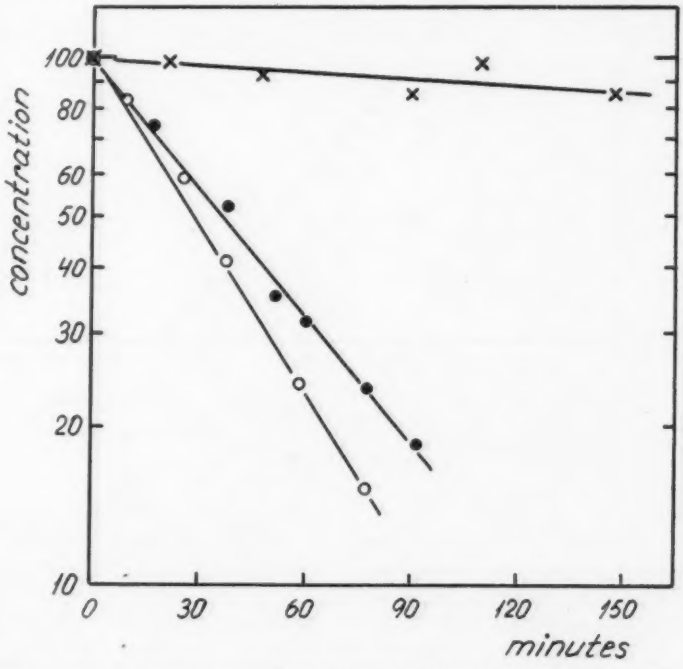

FIGURE 4. *Ciona.* Rate of removal of gastropod (*Haliotis*) hemocyanin (×) and graphite (○ ●).

concentration is plotted against time. The initial concentration is assigned the value of 100. No significant difference was found in the rates of removal of the two materials, even when freshly prepared suspensions of graphite were used. Therefore particles down to 1–2 μ in diameter are effectively removed by *Ciona* from the water passing the branchial basket.

It is also evident from Figure 2 that the curves are nearly straight lines, indicating a constant rate of removal of the suspended material, *i.e.*, a constant rate of water transport throughout the experiments. The relatively constant rate of filtration has always been found in undisturbed, healthy specimens of *Ciona, Mytilus* and *Crassostrea* (see below).

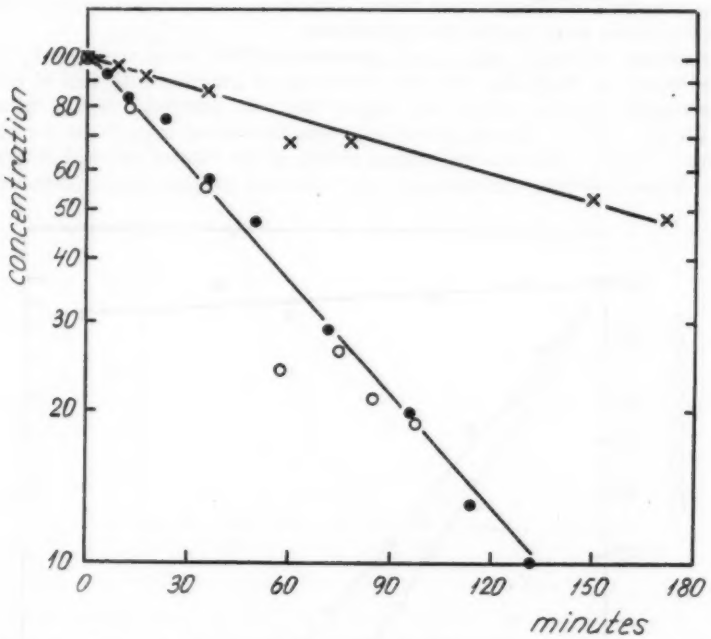

FIGURE 5. *Ciona.* Rate of removal of crab (*Loxorhynchus*) hemocyanin (×) and graphite (○ ●).

In contrast to the effective removal of colloidal graphite, a low degree of retention of the blood proteins was found. In the case of hemoglobin, 90 minutes were required to reduce the hemoglobin concentration by 10%, compared with only three minutes for suspended graphite (Fig. 3). Thus, only a few per cent of the hemoglobin were removed from the water during its passage through the branchial basket. Similar results were obtained with *Haliotis* hemocyanin (Fig. 4), whereas in the only experiment performed with the crab blood, about 50% were extracted in three hours (Fig. 5). More experiments are necessary in order to ascertain

whether this difference is significant in the treatment of *Haliotis* and *Loxorhynchus* blood in the feeding organs of *Ciona*.

Mytilus edulis (L.):

Mytilus edulis were taken from the tidal zone off La Jolla, California. All experiments were performed on the same cluster of four specimens, each of which was about 3 cm. in length. Figure 6 shows that the diatoms were removed somewhat more slowly than the graphite. A partial retraction of the mantle edges was observed, however, upon addition of the diatoms, thus suggesting a depression in the

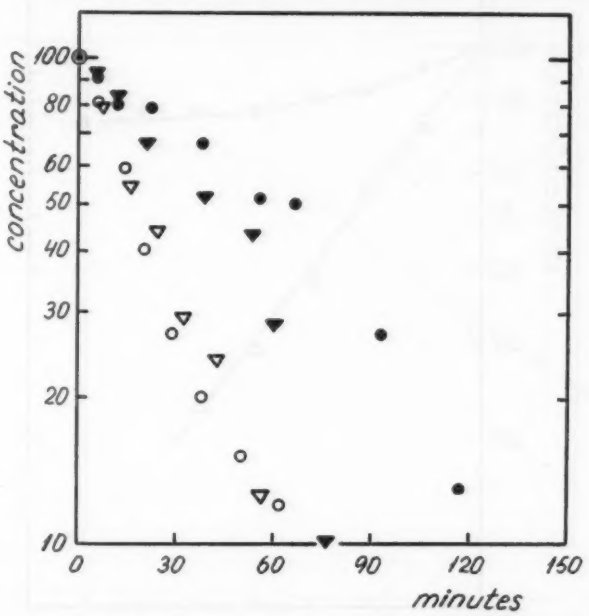

FIGURE 6. *Mytilus edulis*. Rate of removal of diatoms (● ▼) and graphite (○▽).

activity of the mussels. Later in the experiments, the animals again expanded and this coincided with an increased rate of removal of the diatoms which closely corresponded to the removal rates of graphite. Since the colloidal graphite was not removed at a slower rate than the diatoms, the graphite particles were retained practically completely. At the end of the experiments, the graphite concentrations were reduced to about 10% of the initial amount added without any demonstrable decrease in rate of removal. Hence, *Mytilus* efficiently filtered particles at least down to 1–2 μ.

Crassostrea virginica (Gm.):

The oysters used in the present experiments were kindly placed at the disposal of one of us (C. B. J.) by Dr. P. S. Galtsoff of the U. S. Fish and Wildlife Service,

Shellfish Laboratory, Woods Hole, Mass. The animals had been living for about a month in running water in the tanks of the Shellfish Laboratory. The experiments were performed at the Marine Biological Laboratory, Woods Hole. (See also Jørgensen, 1952).

The oysters readily retained all graphite particles in aged suspensions, that is, suspensions more than about one hour old, but removed incompletely the graphite in fresh suspension. Thus, when fresh suspensions of colloidal graphite were offered to the animals, the initial rate of removal was consistently low and decreased

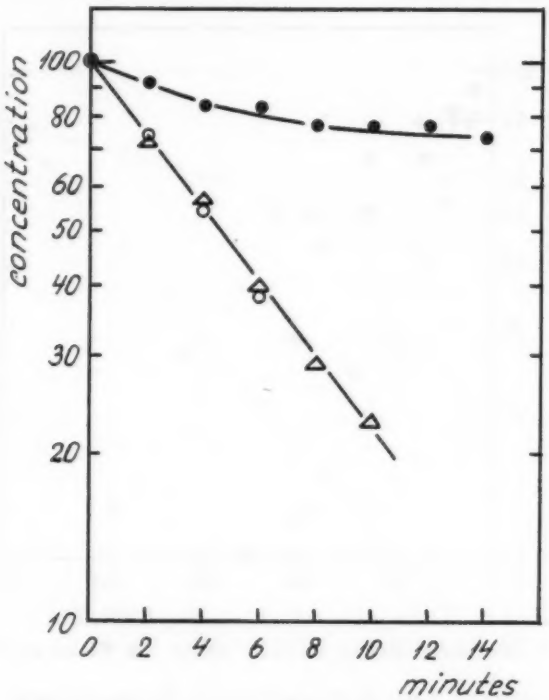

FIGURE 7. *Ostrea virginica.* Rate of removal of graphite from a fresh suspension (●) and from a 4 hours' old suspension (○ △).

with time. Sixteen experiments were carried out, using one oyster at a time. Typical examples are given in Figures 7 and 8. The decreasing removal rates may be due to a preferential retention in the gills of the larger suspended particles causing a decrease in the average size of particles remaining in suspension. Consequently, a less efficient retention in the gill filters resulted. If an aged graphite suspension were exposed to the action of an oyster which was filtering graphite only slowly from a fresh suspension, the rate of removal immediately showed a considerable increase (Fig. 8). In aged graphite suspensions, wherein most particles were

about 2–3 μ in diameter, removal rates were high and constant in 26 experiments. Hence, either all the particles or constant fractions were filtered from the water passing the gills. However, it is difficult to understand why a constant fraction of particles of different sizes should be retained when the dominant size was about 2–3 μ but not when the dominant size was about 1–2 μ as in fresh suspensions. Also, the rates of water transport which could be computed from the removal rates of graphite were high in the experiments with aged graphite suspensions, thus indicating a high degree of retention of graphite particles (Jørgensen, 1952). It must therefore be assumed that particles 2–3 μ in diameter and larger were completely retained by the gill filter under the experimental conditions. The graphite in fresh suspension was

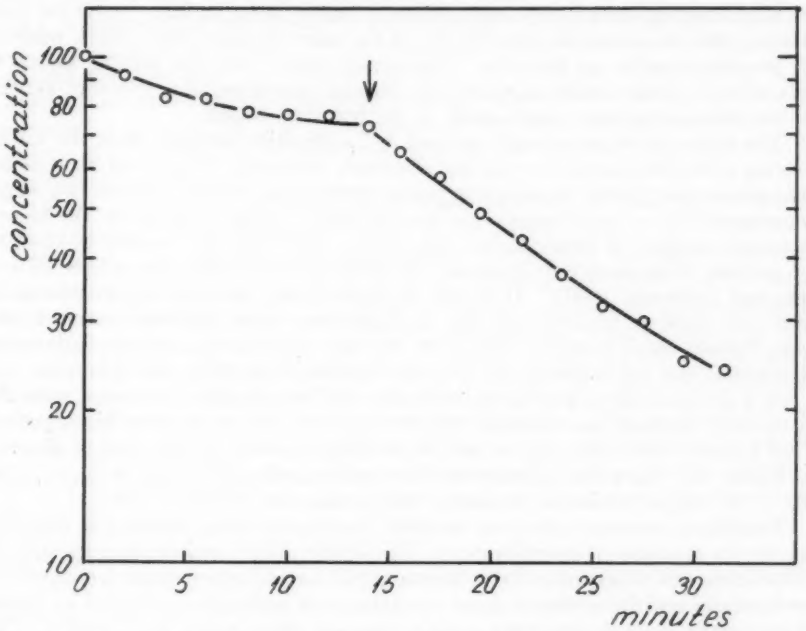

FIGURE 8. *Ostrea*. Rate of removal of graphite from a fresh suspension. At the arrow addition of an aged graphite suspension.

removed about 5 to 10 times more slowly than the graphite in aged suspensions, showing that fewer than 10–20% of the 1–2 μ particles were retained by the mucus of the oyster gills.

DISCUSSION

MacGinitie (1939) observed that when an ascidian is feeding, a sheet of mucus covers the inside of the branchial basket. This mucus is continuously produced by the endostyle and is carried by cilia to the dorsal food groove which conducts the food-loaded mucus toward the mouth (Orton, 1913; Plough and Jones, 1939).

The presence of the mucus sheet can be nicely visualized in transparent *Ciona intestinalis* by addition of colloidal graphite to the water. When *Ciona* is disturbed no feeding mucus is formed and practically no graphite is retained. In the undisturbed animals, however, blackening of the branchial basket results from addition of graphite suspension. The coloring increases in intensity from the region of the endostyle toward the dorsal groove, as should be expected because the mucus sheet is continually carried dorsally. If excessive amounts of graphite, *e.g.*, several milligrams per liter, are added to the water, the animals presently react by closing the exhalent aperture and contracting the body walls violently, thus ejecting water and mucus incorporated with graphite, through the inhalent aperture. The water present in the chamber between the body walls and the branchial basket is forced through the ostia, carrying with it the mucus which is loaded with graphite. After the contraction, this mucus can be seen floating in the water as large black flakes, whereas no graphite remains on the gills. This clearly shows that the feeding mucus is present as a rather stable, continuously flowing membrane-like structure carried by the transporting cilia of the inside of the branchial basket.

The mucus sheet completely strained 1–2 μ graphite particles from the water. Protein molecules, however, were not effectively retained. More than 90% of the hemoglobin and *Haliotis* hemocyanin passed through the mucus, whereas in a single experiment 25% of crab hemocyanin was retained. In the Gastropoda, the known molecular weights of hemocyanins range from more than one million to close to ten millions, whereas in the Crustacea, the values are lower than one million (Svedberg and Pedersen, 1940). It is thus anomalous that the only experiment made with crab blood proteins showed that the latter were more efficiently retained than were *Haliotis* blood proteins. However, the very big hemocyanins met with in the Gastropoda and other groups are known to dissociate in dilute salt solutions. As a rule it is impossible to predict the molecular size in such dilute solutions, since the dissociation products have been investigated only for a few of the giant hemocyanins. They appear to be stable only around the isoelectric points, *i.e.*, at a pH of about 5. At higher pH values they disintegrate into smaller units which may be ¼ to ⅛ the size of the original molecule (Brohult, 1947; Polson and Wyckoff, 1947).

In addition to observations on ascidians, feeding by mucus sheets has been observed, for instance, in lamellibranchs (MacGinitie, 1941) and in some gastropods (MacGinitie and MacGinitie, 1949; Werner, 1951). In the polychaete *Chaetopterus variopedatus* and the echiuroid worm *Urechis caupo* feeding is performed by means of mucus nets through which the water is pumped (MacGinitie, 1937, 1945). The particle-retaining properties of mucus sheets are apparently different in the different types of filter feeders. MacGinitie (1945) thus found that the mucus comprising the feeding nets of *Urechis* and *Chaetopterus* was unable to retain ovalbumin (molecular weight = 44,000), whereas human serum globulin (molecular weight = 176,000) was partially stopped and *Palinurus* hemocyanin (molecular weight = 450,000) was completely stopped in the mucus nets. These nets are therefore probably less effective than the mucus filter of *Ciona* which only incompletely retained the hemocyanins.

When the gastropod *Crepidula fornicata* is feeding, two sets of mucus filters are formed (Werner, 1951). One is guarding the entrance to the mantle cavity and is mainly removing large particles from the inspired water. Smaller particles

pass through, but are strained by the second mucus layer which covers the gills. The dependency of retention on particle size in the two mucus sheets has not been directly measured.

The retention of particulate matter by filter feeding organisms may be a function not only of particle size (reflecting the "porosity of the mucus") but also the charge of the particle relative to the charge of the mucus and possibly of the shape of the particles. Particles with a charge opposite to that of the mucus could be effectively adsorbed. The effect of particle shape and charge on filtering efficiency must await future experimentation.

Lamellibranchia: In *Crassostrea virginica* it was found that graphite particles which were 2–3 μ in diameter were effectively retained by the gills whereas most of the particles about half this size passed through. This is in contrast to the results of Loosanoff and Engle (1947), who found little correlation between particle size and the percentage of particles removed by the gills. The uptake of 5 μ *Chlorella* cells varied from 0 to 92% and of 60 μ *Euglena* cells from 15 to 80%. The concentration of cells was directly measured in the water entering and leaving the oysters. The two bodies of water were separated by a rubber cone enclosing the excurrent side of the oyster.

The properties of the gill filters under these experimental conditions were thus quite different from the properties found in our experiments. The difference in results may be explained by assuming that the oysters were filtering by means of mucus in our experiments but not in those of Loosanoff and Engle. If the mucus sheet were absent, the size of the gill ostia would contribute in determining the degree of retention of particles. Several mechanisms are known which may alter the width of the interfilamental spaces in the gills of Lamellibranchia. Longitudinal muscles in the gill axis and elsewhere can cause a contraction of the entire gill with subsequent narrowing of the slits between the filaments. Vertical muscles in the filaments can produce a shortening and thickening of these with similar effects in the interfilament space. The amount of muscle fibers in the gills varies from species to species (*cf.* Atkins, 1943). If mechanisms of this kind are at work it can be understood how the retention of even large particles can vary from almost complete to practically no retention.

On the other hand, the nearly complete straining, in our experiments, of graphite particles which were only half the size of the smallest cells used by Loosanoff and Engle probably means that mucus sheets, and not the gills proper, performed the straining. Hence, the mucus filtering process in *Crassostrea virginica* effectively retains particles down to about 2–3 μ, only. Similarly, it can be assumed that *Mytilus edulis* was producing mucus sheets in our experiments, and further, that the mucus filter is less porous in *Mytilus* and in *Ostrea* since even 1–2 μ particles were effectively filtered. In previous experiments on *Mytilus edulis* from Plymouth it was observed that the smallest graphite particles generally passed through unadsorbed. This difference between *Mytilus* from California and from Plymouth may be due to differences of specimens from the two localities or due to difference in experimental conditions. From the experiments of Rao (1953) on the variation of pumping rate of *Mytilus californianus* with the latitude, the former explanation appears more reasonable.

Further experiments are needed in order finally to ascertain to what extent formation of mucus filters or adjustment of the size of the interfilamental space without mucus sheet formation are responsible for the effective straining of particles which are only about one or a few microns large.

Summary

1. The efficiency of retention of particles of different sizes has been determined in *Ciona intestinalis, Mytilus edulis* and *Crassostrea virginica* by comparing the rates at which the suspended material was removed from water.

2. The mucus sheet of *Ciona* effectively retained 1–2 μ graphite particles. Only a few per cent of protein molecules such as hemoglobin or gastropod (*Haliotis*) hemocyanin were removed from the water passing through *Ciona*. In one experiment with crab (*Loxorhynchus*) hemocyanin, 25% were retained.

3. *Mytilus* strained practically completely 1–2 μ graphite particles. In *Crassostrea* 2–3 μ particles were effectively filtered from the water, whereas 1–2 μ particles passed through the gills.

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OBSERVATIONS ON MACROPHAGE BEHAVIOR IN THE FIN OF XENOPUS LARVAE

H. E. LEHMAN [1]

Zoologisches Institut, Universität Bern, and the Department of Zoology, University of North Carolina, Chapel Hill, N. C.

In a recent study by Niu and Twitty (1950) which dealt with the origin of epidermal melanophores during metamorphosis in the California salamander *Triturus torosus*, the following observations were made. Dermal melanophores which constitute the primary melanophore population during larval stages were observed to undergo autonomous disintegration shortly before metamorphosis. Phagocytic cells which gave the typical staining reactions for macrophages were observed to move into the dermis and ingest the fragments of disintegrating melanophores. Some of these cells, termed "melanophages," were subsequently seen to differentiate into typical melanophores which took their position in the epidermis and were thereafter indistinguishable from other epidermal melanophores in the skin. The conclusion to this paper contained a suggestion which was of particular interest: namely, "that the course of cellular differentiation is susceptible to control or modification in accordance with the specific character of substances transmitted or introduced through the cytoplasm" (p. 647).

The present experiments were undertaken for the purpose of examining this phenomenon in another species in which the details of cellular behavior are particularly favorable for observation. For this purpose the larvae of the clawed African toad, *Xenopus laevis* were used (see Weisz, 1945a, 1945b for normal development). In *Xenopus* larvae, the posterior third of the tail is abundantly supplied with melanophores, but the anterior ⅔ of the ventral fin is entirely free of pigment cells. In this pigment-free region, the fin is very thin and completely transparent so that the details of cell activity can be observed with great clarity.

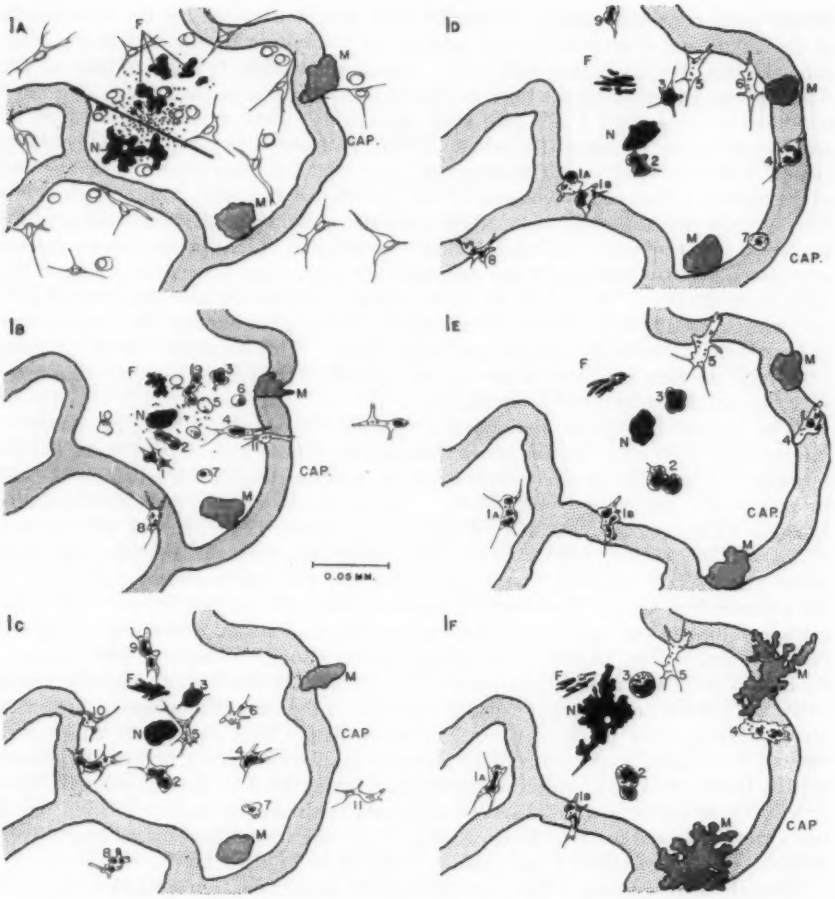
The animals used in the following study were derived from the *Xenopus* colony maintained at the Zoologisches Institut, Bern. The eggs were obtained by hormonally induced spawning following the injection of estrogenic hormones into the dorsal lymph space of mature male and female animals. The method was that of Gasche (1943) and modified by Andres *et al.* (1948). The eggs were permitted to develop until the feeding stage and thereafter the larvae were fed a suspension of nettle powder as recommended by Gasche (1943, 1944). Larvae ranging in length from 10 to 20 mm. were used in the experiments and all operations and observations were carried out on animals narcotized in one part MS 222 (Sandoz) to 7000 to

[1] National Science Foundation post-Doctoral Fellow at the Universität Bern, Sept. 1952–Sept. 1953. Now at the University of North Carolina. The writer takes this opportunity to express his deepest appreciation to Professor Fritz Baltzer for his stimulating interest and critical suggestions, to Professor F. E. Lehmann for the living material and numerous creative ideas, and to the staff of the Zoologisches Institut for their cordial hospitality and technical assistance during the tenure of the fellowship.

10,000 parts aquarium water. A special slide was constructed for the observation of the larvae and it served the dual purpose of preventing drying and at the same time elevating the fin so that high power objectives could be brought to bear on the field without endangering the thick head and trunk regions of the body. This slide consisted of a standard 1 × 3 inch slide upon which were mounted two halves of another slide, one on top of the other. They were sealed in place by deKhotinsky cement which is highly water resistant and excellent for this purpose. At one end of the mounted glass pieces was attached a "U"-shaped glass rod, 3 mm. in diameter. The latter provided a small well to hold narcotizing fluid and the head and trunk of the larva. During periods of observation, a small piece of lens paper one cm. square was placed over the head and trunk and capillarity thereafter prevented drying of the delicate epidermis. The tail of the larva extended on the elevated area of the slide and was covered by a standard 22 mm. square cover glass bearing two small drops of bee's wax at the corners placed nearest the body. This supported the cover glass at one end and permitted it to fit the tapered contour of the tail without applying pressure that would interfere with circulation.

The experimental procedures were as follows. Two cubic mm. of the hormone intermedin (2000 units vairon per cc. Intermédin, Choay Lab. Paris) from the intermediate lobe of the pituitary were injected intramuscularly at the base of the tail by means of a fine glass mouth-pipette. This hormone has the well known ability to cause melanophore expansion (Hogben and Slome, 1931; Zondek and Krohn, 1932a, 1932b; Parker, 1940). Two hours after injection, the animals with their melanophores fully expanded were narcotized and one melanophore at the edge of the pigment-free area of the ventral fin was cut into two unequal halves. One part contained the nucleus, and the other consisted of pseudopodial extensions of the cell body (N and F, Fig. 1A–F). All operations were done free-hand at 100 × magnification by means of a finely sharpened steel needle. A small notch was also made at the edge of the fin to mark the general location of the cut melanophore. A camera lucida drawing was made of the region immediately after operation; this figure showed, in addition to the cut cell, the adjacent capillaries, nerves, and melanophores so that there could be no question concerning the position of the cut cell at a later time. These cut cells were observed daily and re-drawn by camera lucida. Most cases were followed for 7 to 10 days, and a few were maintained for as long as 5½ weeks. One representative case history is given in Figures 1A–F.

Shortly after cutting, a few, and frequently many, melanin granules were released from the pigment cell. These granules were freely dispersed in the jelly matrix of the fin in the vicinity of the cell. The amputated pseudopodia tended to contract slightly during the first day following transection, and during the second day phagocytic cells usually completed the process of engulfing all except the largest cell fragments (Figs. 1A, B, C). The latter were frequently observed to remain intact for over a week. The phagocytes which participated in ingestion of melanophore fragments and pigment granules were of two varieties: 1) spherical or oval cells from the blood stream, which entered the field from adjacent capillaries, and 2) stellate mesenchyme cells *in situ* in the matrix of the fin. The former correspond to the "free macrophages" and the latter to the "fixed macrophages" referred to in the literature on the reticulo-endothelial system (see reviews by Levi, 1934; Chevremont, 1942). Preliminary experiments which in part duplicated the results of Vierling



FIGURES 1A-F. *A case history of macrophage ingestion of fragments of a cut melanophore* (camera lucida tracings). *1A*, after 24 hours; *1B*, 2 days; *1C*, 3 days; *1D*, 4 days; *1E*, 5 days, just before injection of intermedin; *1F*, 2 hours after *1E*. Abbreviations; *cap.*, capillary; *F*, non-nucleated melanophore fragment; *N*, nucleated melanophore fragment; *M*, normal melanophores; *1–11*, macrophages with ingested melanophore fragments or melanin granules.

(1926) showed that both of these cell types had a pronounced tendency to accumulate carmine injected into the fin.

The amount of melanin taken up by a single macrophage varied from a few granules (cells 5, 6, 10, 11, Figs. 1B–F), to such great quantities that the cell appeared to be gorged by the ingested particles (cells 1, 2, 3, 4 and 9). In general, those cells which contained only a moderate amount of melanin retained the particles longer than those containing excessive amounts. Some of the cells with fewer than

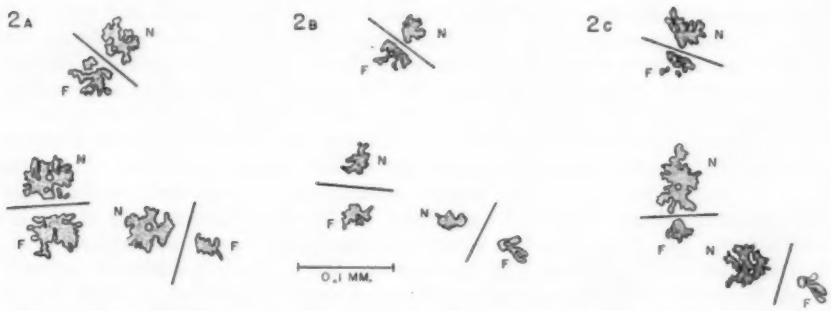
a dozen granules retained these particles for as long as 5 weeks. Macrophages containing large cell fragments usually cast off these particles into the matrix of the fin after 7 to 10 days, or, as was observed in three separate instances, the cells migrated into a capillary and were carried away by the blood stream (cells 7 and 8). The majority of macrophages containing ingested melanin usually remained in the vicinity of the cut melanophore; however, some of these cells showed marked activity (cells 6, 9, 10, and 11) and it was found to be impractical to attempt to follow cells that moved any great distance from the original field.

In none of the cases followed was there any evidence that the macrophages had acquired the ability to synthesize pigment after having phagocytized melanophore fragments. The ingested particles were generally clustered in the interior of the cells in discrete clumps which were quite different in appearance from the arrangement found in normal melanophores. It was not determined whether or not these large aggregations of melanin were contained in vacuoles; however, individual melanin grains appeared to be free in the cytoplasm. Some macrophages with moderate to large amounts of ingested material gave a superficial resemblance to contracted pigment cells (cells 2 and 3) and at first it was thought that these cells might indeed be melanophores in a true sense, since, under usual conditions, the normal melanophores remain in the contracted phase. In order to provide a decisive test to determine whether or not these cells did possess the physiological properties of melanophores, it was decided to see if they would respond in a typical manner to the influence of the hormone intermedin. To this end, four units of the hormone were injected into the base of the tail in the manner already described. Following these injections, not one of the "pseudo-melanophores" responded, whereas host melanophores became expanded maximally, as did the nucleated half of the cut melanophore (see Figs. 1E–F). Since the macrophages gave no response to the hormone, and never gave any indication that they were actively synthesizing melanin, it can be concluded that under the conditions of these experiments, at least, the ingestion of melanin did not stimulate macrophages to become melanophores. In this respect the macrophages of *Xenopus* larvae differed from these observed by Niu and Twitty (1950) in *Triturus torosus*.

It should be borne in mind that the observations of Niu and Twitty were based on normal and not on experimental conditions of behavior. Moreover, the observations on *T. torosus* were made on metamorphosing animals: a time notable for extensive developmental changes which take place under the influence of metamorphosing hormones. The latter fact makes it particularly difficult to compare the results of Niu and Twitty with those on *Xenopus* larvae. Furthermore, it was pointed out by Niu and Twitty that not all macrophages which ingested melanophore fragments in *T. torosus* became converted into melanophores; this particular feature is abundantly confirmed by the present study. Both studies therefore demonstrate that the ingestion of specific types of cellular debris does not play a constant role in determining the fate of the macrophages in question. The only histological transformation of these cells observed in the present experiments was the ready conversion of spherical "free macrophages" from the blood stream into stellate "fixed macrophages" indistinguishable from the mesenchyme cells of the fin matrix (cells 5 and 6). The reciprocal change was not so clearly demonstrable even though some of these stellate cells were observed to migrate into capillaries (cell 8). No clear-cut case

was noted in which a mesenchyme cell assumed the typical, pseudopod-free, spherical, character of those cells which originated in the blood stream. A final consideration which should be kept in mind in attempting to compare the results from *T. torosus* and *Xenopus laevis* is the importance of genetic differences which may well play a very significant part in restricting the developmental capacities and behavior properties of macrophages in the two species.

One additional observation on macrophage behavior in *Xenopus* is included at this time even though it is only indirectly related to the foregoing question of macrophage determination following ingestion of cytoplasmic particles. This concerns differences in the response of nucleated and non-nucleated melanophore fragments to the influence of the hormone intermedin. It was noted that within two or three hours after an expanded melanophore was cut, the influence of injected intermedin to a large measure disappeared (Figs. 2A–B). The nucleated half along with the great majority of host melanophores underwent a contraction of granules into a dense mass around the nucleus. The non-nucleated fragments showed less tendency



FIGURES 2A–C. The influence of intermedin on nucleated and non-nucleated fragments of melanophores (camera lucida tracings). *2A*, 3 melanophores expanded by an injection of intermedin 2 hours previously and drawn immediately after cutting (*N*, nucleated half; *F*, non-nucleated fragment); *2B*, the same cells 2 hours later, after contraction of melanin has begun and just before a second injection of intermedin; *2C*, the same cells 2 hours after the second injection of intermedin; note that only the nucleated fragments have expanded after cutting.

to contract. When intermedin was again injected into these animals two hours after cutting, the nucleated fragments and the normal cells quickly expanded, whereas the non-nucleated parts remained unchanged (Figs. 2B and 2C). These preliminary observations suggest that the cytoplasm of the melanophore requires the presence of the nucleus in order to respond to the expanding stimulus of the hormone intermedin. In any event it would appear that the ability to respond to the hormone is lost by the cytoplasm within the first four hours after separation from the nucleus.

SUMMARY

1. The hormone intermedin was injected into *Xenopus* larvae to induce pigment cell expansion. In the expanded condition melanophores were cut and macrophage ingestion of melanin granules and cell fragments was observed.

2. Macrophages retained parts of the ingested pigment cells for from one to five weeks. However, in no instance were these macrophages observed to synthesize melanin or to exhibit the physiological characteristics of melanophores as judged by their responsiveness to intermedin.

3. Preliminary observations on the influence of intermedin on nucleated and non-nucleated fragments of melanophores suggest that the cytoplasm of these pigment cells requires the presence of the nucleus in order to respond to the expanding stimulus of this hormone.

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INDEX

ATP as energy source in cell division, 366.
Activation, mechanism of in Chaetopterus egg, 87.
Activation of Spisula egg, 213.
Adenosine triphosphate, dephosphorylation of by tissues of cockroach, 166.
Aging, physiological, in adult worker honey bee, 154.
Alga, photosynthesis and phototaxis in gametes of, 103.
Algae as food for Balanus larvae, 420.
Algae, Vaucheriae, in New England salt marshes, 395.
ALLEN, ROBERT DAY. Fertilization and artificial activation in the egg of the surf-clam, Spisula solidissima, 213.
Amino acid content of Teredo, 316.
Amino acids, oxidation of by marine animals, 390.
Amoebocytes of quahog, cholinesterase and lipase in, 200.
Amphibian gastrulae, reactions of to metabolic poisons in vitro, 466.
Amphibian larvae, Xenopus laevis, macrophage behavior in, 490.
ANDERSON, JOHN D., AND C. L. PROSSER. Osmoregulating capacity in populations occurring in different salinities, 369.
ANDERSON, JOHN MAXWELL. Structure and function in the pyloric caeca of Asterias forbesi, 47.
Annelid, effect of x-rays on anterior regeneration in, 80.
Annelid egg, mechanism of activation and nuclear breakdown in, 87.
Annelid, permeability of coelomic fluid cells of, 128.
Annelid, ploychaetous, from Lake Erie, 149.
Annual Report of the Marine Biological Laboratory, 1.
Anterior regeneration in Aulophorus, as affected by x-rays, 80.
Anticoagulant, release of from irradiated Spisula eggs, 380.
Antimitotic substances in ovaries, 375.
Antimitotic substance in puffer ovary, 372.
Apis mellifera, physiological aging in, 154.
Apyrase activity of squid mantle muscle, 392.
Aqueous phase, chemoreception in, 115.
Ascidians, particle filtration in, 477.
Aspects of physiological aging in adult worker honey bee, 154.
Assay of growth and differentiation hormone of Lepidoptera by tissue culture, 174.
Asterias forbesi, structure and function in pyloric caeca of, 47.
Aulophorus furcatus, regeneration of anterior end of, 80.
Axone, squid, elastic and ionic studies on, 370.
Axoplasm of squid giant axone, structure of as revealed by spontaneously formed vesicles, 376.

BARNACLE, growth and molting in, 420.
BARNES, LOY J. A further study of the effects of centrifugation and low temperature on the development of Fundulus heteroclitus, 370.
Basal metabolism, tissue repiration and growth, 240.
Bats, feeding efficiency of, 364.
Beetle, chemoreception in aqueous and gas phases by, 115.
BENNETT, MIRIAM F. *See* H. MARGUERITE WEBB, 386; FRANK A. BROWN, Jr., 371.
VON BERTALANFFY, L., AND W. J. PIROZYNSKI. Tissue respiration, growth, and basal metabolism, 240.
Bird testes, roles of darkness and light in photoperiodic response of, 434.
BLOCH, DONALD I. *See* BERTRAM SACKTOR, 166.
BLUM, JOHN L., AND JOHN T. CONOVER. New or noteworthy Vaucheriae from New England salt marshes, 395.
Body size and tissue respiration in rat, 240.
Body weight and body surface area in marine fishes, 285.
Bombyx mori, studies on integument of, 308.
BOOKHOUT, C. G. *See* JOHN D. COSTLOW, JR., 420.
BOYARSKY, L. Elastic and ionic studies with the squid giant axone, 370.
Breakdown of nucleus in Chaetopterus egg, mechanism of, 87.
BRILL, ARTHUR S., AND JOHN M. OLSON. Diffusion coefficient of hemerythrin, 371.
BROOKES, VICTOR J. *See* G. FRAENKEL, 442.

BROWN, FRANK A., JR. A new type of respirometer which permits continuous and automatic recording of metabolic rate, 362.
BROWN, FRANK A., JR. *See* MURIEL I. SANDEEN, 381.
BROWN, FRANK A., JR., MIRIAM F. BENNETT AND MURIEL I. SANDEEN. Temperature independence of the frequency of the endogenous tidal rhythmicity of the fiddler crab, Uca pugnax, 371.
BROWN, FRANK A., JR., MIRIAM F. BENNETT AND H. MARGUERITE WEBB. Endogenously regulated diurnal and tidal rhythms in metabolic rate in Uca pugnax, 371.
BUCK, JOHN B., AND MARGARET L. KEISTER. Cutaneous and tracheal respiration in the Phormia larva, 402.
BUZZELL, ANNE. *See* MAX A. LAUFFER, 357.

CAECA, pyloric, structure and function of, in Asterias, 47.
Calcium, effect of on proteins of sea urchin egg homogenates, 365.
Calcium ion, action of in activating breakdown of ATP by cockroach tissues, 166.
Calcium, role of in activation and nuclear breakdown of Chaetopterus egg, 87.
Calcium, role of in fertilization and artificial activation of Spisula egg, 213.
Carbohydrate metabolism of developing Chaetopterus egg, 359.
Carbon dioxide production by diapausing silkworm pupae, 382.
Carbon dioxide, permeability of silkworm integument to, 308.
Carotenoid content of Ulva lactuca thallus, 103.
Carotenoid, possible presence and synthesis of in Tubularia, 450.
Cation relationships at Fundulus ectoderm, 368.
Cecropia silkworm, in vitro spermatogenesis of, 174, 188.
Cecropia silkworm, metabolism of during diapause and development, 320.
Cells, erythrocyte-like, of Phascolosoma, permeability of, 128.
Centrifugal force, effect of on development of Chaetopterus eggs, 389.
Centrifugation and development of Fundulus eggs, 370.
Centrifugation of Tetrahymena, 133.
Centrifuged Arbacia egg, role of vitelline membrane in fragmentation of, 384.
Chaetopterus egg, mechanism of activation and nuclear breakdown in, 87.
CHAPMAN, KENT M. Colliding impulse studies of the relation between longitudinal transport and afterpotential in nerve of frog and squid, 362.
Chemoreception in aqueous and gas phases, 115.
Chemoreceptors of blowfly, stimulation of, 257.
CHENEY, RALPH HOLT, AND A. I. LANSING. Surface structure limitation of fertilization, 363.
CHILD, C. M. Redox indicator patterns in relation to echinoderm exogastrulation. I. Oxidation patterns, 62.
Cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle of quahog, 200.
Ciliary activity in Mytilus gill, 378.
Ciona intestinalis, particle filtration in, 477.
CLARKE, EDITH C. *See* ERIC G. BALL, 369.
Cleavage of Arbacia eggs, effects of some antibiotics on, 378.
CLENDENNING, K. A. *See* F. T. HAXO, 103.
Clotting of perivisceral fluid of sand dollar, 372.
CLOWES, G. H. A. *See* M. E. KRAHL, 377.
Cockroach, dephosphorylation of ATP by tissues of, 166.
Coelenterate reconstitution, pigment synthesis during, 450.
Coelomic cells of Phascolosoma, permeability of, 128.
Coenosarc, role of in synthesizing pigment during Tubularia reconstitution, 450.
COHEN, MELVIN J. Oscillographic analysis of an invertebrate equilibrium organ, 363.
Colloidal changes during aging of Chaetopterus egg, 388.
COMITA, JEAN J., AND ARTHUR H. WHITELEY. The correlation of desoxyribonucleic acid synthesis and the rate of respiration in the sea urchin embryo, 412.
Conjugation in Tetrahymena, 269.
Conjugation in Tetrahymena, nucleo-cytoplasmic interaction during, 133.
CONOVER, JOHN T. *See* JOHN L. BLUM, 395.
Contralateral stimulation of blowfly tarsal chemoreceptors, 257.
COOPER, OCTAVIA. *See* ERIC G. BALL, 369.
Correlation of DNA synthesis and respiration rate of sea urchin embryo, 412.
Cortical changes in Chaetopterus egg, 87.
COSTLOW, JOHN D., JR., AND C. G. BOOKHOUT. Moulting and growth in Balanus improvisus, 420.
COUILLARD, PIERRE. An antimitotic substance in the ovary of the common puffer, Sphaeroides maculatus, 372.
Crassostrea virginica, particle filtration in, 477.

CROWELL, SEARS. The regression-replacement cycle of hydranths in Obelia and Campanularia, 355.
Cutaneous and tracheal respiration in the Phormia larva, 402.
Cuticle of silkworm, studies on, 308.
Cyanide, action of on protoplasmic colloid, 388.
Cytology of conjugation in Tetrahymena, 269.
Cytoplasmic-nuclear interaction during conjugation in Tetrahymena, 133.

DDT, effects of on respiration of Phormia larva, 402.
DNA content of cells, determination of, 354.
DNA synthesis, correlation of with respiration rate in sea urchin embryo, 412.
Darkness, role of in photoperiodic response of testes of white-crowned sparrows, 434.
DAVIDSON, ERIC. Clotting of the perivisceral fluid of the sand dollar, Echinarachnius parma, 372.
Dendraster excentricus, oxidation patterns in exogastrulae of, 62.
Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, Periplaneta americana (L.), 166.
DETHIER, V. G. Summation and inhibition following contralateral stimulation of the tarsal chemoreceptors of the blowfly, 257.
Development of lordotic and normal guppy, 160.
Development of R. pipiens eggs, effects of elliptically polarized electric currents on, 384.
Development of silkworm, respiratory metabolism during, 320.
Development of surf-clam egg, 213.
Diapause, physiology of, 174, 188, 320.
Differentiation and growth hormone of Lepidoptera, assay of by tissue culture method, 174.
Digestion in Asterias, 47.
DILLER, IRENE COREY. Effect of aqueous extract of beef spleen on cells of sarcoma 37 ascites tumor, 363.
Distribution of Neanthes (Nereis) in relation to salinity, 335.
Distribution of nitrogen in Teredo, 316.
Distribution of pentose nucleic acid in gastrula and neurula of Triturus, 348.
Diurnal rhythms of oxygen consumption in Littorina and Urosalpinx, 381.
Drosophila melanogaster, means of adhesion of puparia of to substrate, 442.
DUNN, ARNOLD. Some properties of the mitotic inhibitor in starfish ovary extracts, 373.

ECHINOCHROME granules, motion of in fertilized Arbacia egg, 368.
Echinoderm exogastrulae, indicator patterns in, 62.
Echinoderm, structure and function of pyloric caeca in, 47.
Ecological studies on Neanthes, 335.
Effect of x-rays on anterior regeneration in Aulophorus, 80.
Effects of temperature, oxygen tension and metabolic inhibitors on in vitro spermatogenesis in Cecropia silkworm, 188.
Egg, Chaetopterus, mechanism of activation and nuclear breakdown in, 87.
Egg, surf-clam, fertilization and artificial activation in, 213.
EICHEL, HERBERT J., AND JAY S. ROTH. Effects of x-radiation on oxidative systems of Tetrahymena pyriformis, 373.
Elasmobranchs, isotonic solutions for erythrocytes of, 289.
ELLIOTT ALFRED M., AND ROBERT E. HAYES. Mating types in Tetrahymena, 269.
Embryos, amphibian, reactions of to metabolic poisons in vitro, 466.
Embryo, sea urchin, correlation of DNA synthesis with respiration of, 412.
Embryonic development of lordotic and normal guppy, Lebistes reticulatus (Peters), 160.
Equilibrium organ, invertebrate oscillographic analysis of, 363.
Erythrocytes of marine teleosts and elasmobranchs, isotonic solutions for, 289.
Erythrocyte-like cells of Phascolosoma, permeability of, 128.
Excretion of P-32 by Habrobracon females, 296.
Exogastrulation, indicator patterns in, 62.
Explant systems and reactions of gastrulating amphibians to metabolic poisons, 466.

FARNER, DONALD S., L. R. MEWALDT AND STANLEY D. IRVING. The roles of darkness and light in the photoperiodic response of the testes of white-crowned sparrows, 434.
Fate of radiophosphorus ingested by Habrobracon females, 296.
Feeding of ascidians and lamellibranchs, 477.
Fertilization and artificial activation in egg of surf-clam, Spisula (Mactra), 213.
Fertilization, surface structure limitation of, 363.
Fibrinogen, clotting of as a problem of protein chemistry, 356.
Filtration of particles by ascidians and lamellibranchs, 477.
Fish, development of, 160.

Fishes, marine, relation of body weight to body surface area in, 285.
Flies (Phormia), stimulation of tarsal chemoreceptors of, 257.
Fly puparia, means of attachment of to substrate, 442.
FOULKES, ROBERT HUGH. Regeneration of the anterior end of Aulophorus furcatus (Naididae) with special reference to effect of x-rays, 80.
FRAENKEL, G., AND VICTOR J. BROOKES. The process by which the puparia of many species of flies become fixed to a substrate, 442.
Fresh-water polychaetous annelid, Manayunkia speciosa, from Lake Erie, 149.
FROST, J. L. *See* F. B. BANG, 361.
Function of pyloric caeca of Asterias, 47.
Fundulus, anomalous embryo of, 382.

GAMETES of Ulva lactuca, photosynthesis and phototaxis in, 103.
Gas phase, chemoreception in, 115.
Gastrula, distribution of pentose nucleic acid in, 348.
Gastrulating amphibians, reactions of to metabolic poisons in vitro, 466.
GEVIRTZ, NORMAN R. *See* HERMAN M. KALCKAR, 391.
Glutathione, hydrolysis and transpeptidation of in marine forms, 369.
Glycolysis and potassium-sodium exchange in rabbit red cells exposed to ethyl and *n*-propyl carbamate, 357.
GOLDBERG, EDWARD D. *See* C. BARKER JØRGENSEN, 477.
GOLDMAN, ALLEN S. Synthesis of pigment during the reconstitution of Tubularia, 450.
GOLDSTEIN, LESTER. A study of the mechanism of activation and nuclear breakdown in the Chaetopterus egg, 87.
GOLDSTEIN, LESTER, AND NORMA O. GOLDSTEIN. A study of some proteolytic factors in the unfertilized Chaetopterus egg, 374.
Gonads, roles of darkness and light in photoperiodic response of, in birds, 434.
GOULD, EDWIN. Feeding efficiency of bats, 364.
GRAVES, ROBERT S. *See* H. MARGUERITE WEBB, 386.
GRAY, I. E. Relation of body weight to body surface area in marine fishes, 285.
GREEN, JAMES W., AND JOSEPH F. HOFFMAN. A study of isotonic solutions for the erythrocytes of some marine teleosts and elasmobranchs, 289.
GREEN, J. W., AND JAY S. ROTH. The effect of S-35 on the development of Arbacia eggs, 364.
GREEN, J. W., AND F. X. WAZETER. Glycolysis and potassium-sodium exchange in rabbit red cells exposed to ethyl and *n*-propyl carbamate, 357.
GREGG, JOHN R., AND NORMA ORNSTEIN. Explant systems and the reactions of gastrulating amphibians to metabolic poisons, 466.
GROSCH, DANIEL S., AND ROBERT L. SULLIVAN. The fate of radiophosphorus ingested by Habrobracon females, 296.
GROSS, PAUL R. The effect of calcium on the proteins of sea urchin egg homogenates, 365.
Growth and differentiation hormone of Lepidoptera, assay of by tissue culture method, 174.
Growth and moulting in Balanus, 420.
Growth, tissue respiration, and basal metabolism, 240.
Guppy, development of lordotic and normal, 160.

HABROBRACON females, ingestion of radiophosphorus by, 296.
HAMER, D. Isolation of protein fractions from echinoderm sperms and eggs, 389.
HAMER, D. Oxidation of amino acids by marine animals, 390.
HAMMOND, WARNER S. Neuronal derivatives of the neural crest in the chick, 355.
HARVEY, ETHEL BROWNE. A simplified electrical method of determining the sex of sea urchins and other marine animals, 365.
HAYWARD, HUGH R. *See* GEORGE T. SCOTT, 358.
HAYWARD, HUGH R., AND GEORGE T. SCOTT. Evidence for independent mechanisms regulating potassium accumulation and sodium secretion in Ulva lactuca, 366.
HAXO, F. T., AND K. A. CLENDENNING. Photosynthesis and phototaxis in Ulva lactuca gametes, 103.
HAXO, F. T., AND PHYLLIS S. NORRIS. Photosynthetic activity of phycobilans in some red and blue-green algae, 374.
HAYES, ROBERT. *See* ALFRED M. ELLIOTT, 269.
Heart muscle of quahogs, cholinesterase and lipase in, 200.
HEILBRUNN, L. V. *See* WALTER L. WILSON, 388.
HEILBRUNN, L. V., AND WALTER L. WILSON. The action of parthenogenetic agents on

the interior protoplasm of the Chaetopterus egg, 375.
HEILBRUNN, L. V., WALTER L. WILSON AND MURIEL LIPPMAN. Antimitotic substances present in ovaries, 375.
Hemerythrin, diffusion coefficient of, 371.
Hemerythrin, effect of sulfide-blocking reagent on oxygenation of, 377.
"Hemolysis" of coelomic fluid cells of Phascolosoma, 128.
HEMPLING, H. G. Potassium and sodium movements in rabbit polymorphonuclear leukocytes, 356.
Hexokinase and isomerase activity in Arbacia eggs, 377.
HILD, WALTHER. Elaboration of so-called posterior lobe hormones in the hypothalamus, 360.
HINES, MARGARET N. Use of autotomized legs in determining the phases of the tidal rhythm in Uca pugnax, 375.
Histochemistry of quahog tissues, 200.
Histochemistry of starfish pyloric caeca, 47.
HODGSON, EDWARD S. A study of chemoreception in aqueous and gas phases, 115.
HOFFMAN, JOSEPH F. *See* JAMES W. GREEN, 289.
Homarus ganglion, integration in, 367.
Honey bees, physiological aging in, 154.
Hormone, growth and differentiation, assay of in Lepidoptera by tissue culture method, 174.
HUXLEY, A. F. Observations with an interference microscope, 390.
Hydranth regression-replacement cycle in Obelia and Campanularia, 355.
Hypothalamus as source of elaboration of "posterior lobe hormones," 360.

IN vitro assay of Lepidopteran growth and differentiation hormones, 174, 188.
Indicator patterns in echinoderm exogastrulae, 62.
Ingestion of radiophosphorus by Habrobracon females, 296.
Inhibition and summation after contralateral stimulation of blowfly tarsal chemoreceptors, 257.
Inhibitors, metabolic, effect of on in vitro spermatogenesis of Cecropia silkworm, 188.
Insect, Phormia, cutaneous and tracheal respiration in, 402.
Insect, Phormia, stimulation of tarsal chemoreceptors of, 257.
Insect diapause, physiology of, 174, 188, 320.
Insulin sulphate, acid, possible globulite structure for, 389.
Integument of silkworm, studies on, 308.
Interaction of nucleus and cytoplasm during conjugation in Tetrahymena, 133.
Interference microscopy of cells, 390.
Intermedin, effect of on melanophores of Xenopus larva, 490.
Intestinal epithelium of quahog, cholinesterase and lipase in, 200.
Irradiation, effect of on anterior regeneration in Aulophorus, 80.
IRVING, STANLEY D. *See* DONALD S. FARNER, 434.
Isolated blastomeres of Fundulus, development of, 383.
Isotonic solutions for marine teleost and elasmobranch erythrocytes, 289.
Isotopes (P-32), effect of ingested, 296.
ITO, TOSHIO. Studies on the integument of the silkworm, Bombyx mori. VII. The permeability of the integument to oxygen and carbon dioxide in vivo, 308.

JACOB, MIRIAM I. Physiological studies of centrifuged normal and carcinogen-treated Tetrahymena, 376.
JOHNSON, THOMAS N. The effects of various acids on the receptors of the amphibian tongue, 391.
JØRGENSEN, C. BARKER, AND EDWARD D. GOLDBERG. Particle filtration in some ascidians and lamellibranchs, 477.

KALCKAR, HERMAN M., JACK L. STROMINGER AND NORMAN GEVIRTZ. A study of an unidentified ultraviolet absorbing substance occurring in the tissues of marine animals, 391.
KAO, CHIEN-YUAN. Structure of axoplasm as revealed by spontaneously formed vesicles, 376.
KAYE, ALVIN M. *See* PETER RIESER, 380.
KEISTER, MARGARET L. *See* JOHN B. BUCK, 402.
KELTCH, A. K. *See* M. E. KRAHL, 377.
KETCHEL, MELVIN. *See* HOWARD A. SCHNEIDERMAN, 188.
KLOTZ, I. M., S. RAPAPORT AND E. V. H. ROSENBERG. The effect of a sulfide-blocking reagent on the oxygenation of hemerythrin, 377.
KRAHL, M. E., A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES. Hexokinase and isomerase activity in eggs of the sea urchin, Arbacia punctulata, and other marine forms, 377.

LACCOPHILUS maculosus, chemoreception in aqueous and gas phases by, 115.
Lamellibranchs, particle filtration in, 477.
LANDAU, JOSEPH. *See* DOUGLAS MARSLAND, 366.
LANE, CHARLES E. *See* REUBEN LASKER, 316.
LANSING, A. I. *See* RALPH HOLT CHENEY, 363.
LAPLACE, RUTH. *See* CLAUDE VILLEE, 386.
Larva, amphibian, behavior of macrophages in fin of, 490.
Larva, Phormia, cutaneous and tracheal respiration in, 402.
LASKER, REUBEN, AND CHARLES E. LANE. The origin and distribution of nitrogen in Teredo bartschi Clapp, 316.
Lateral line receptor cell, specific pigment in, 386.
LAUFFER, MAX A., ANNE BUZZELL AND DAVID TRKULA. Thermal inactivation of a bacterium-bacteriophage system, 357.
Lebistes reticulatus (Peters), development of lordotic and normal, 160.
LEHMAN, H. E. Observations on macrophage behavior in the fin of Xenopus larvae, 490.
Lepidoptera, assay of growth and differentiation hormone of by tissue culture method, 174.
Light, role of in photoperiodic response of testes of white-crowned sparrows, 434.
Limulus, toxic effect of marine bacterium on, 361.
Lipase in amoebocytes, intestinal epithelium and heart muscle of quahog, 200.
LIPPMAN, MURIEL. *See* L. V. HEILBRUNN, 375.
Longitudinal transport, colliding impulse studies of, 362.
LORAND, L. Clotting of fibrinogen as a problem of protein chemistry, 356.
Lordotic guppy, development of, 160.
LOVE, WARNER E. The permeability of the erythrocyte-like cells of Phascolosoma gouldi, 128.
Lysis of coelomic cells of Phascolosoma, 128.

MACROPHAGE behavior in Xenopus larval fin, 490.
Mactra (Spisula) egg, fertilization and artificial activation of, 213.
Magnesium ion, action of in activating breakdown of ATP by cockroach tissues, 166.
Manayunkia speciosa, from Lake Erie, 149.
Marine fishes, relation of body weight to body surface area in, 285.
Marine teleosts and elasmobranchs, isotonic solutions for, 289.
MARONEY, SAMUEL P., JR., AND R. R. RONKIN. Cholinesterase and ciliary activity in the gill of Mytilus, 378.
MARSLAND, DOUGLAS, JOSEPH LANDAU AND ARTHUR ZIMMERMAN. Adenosine triphosphate as an energy source in cell division; pressure-temperature experiments on the cleaving eggs of Arbacia and Chaetopterus, 366.
Mating types in Tetrahymena, 269.
MAURO, A. The electrochemical potential difference of the chloride ion in the giant squid axon-sea water system, 378.
MAYNARD, DONALD M. Integration in the cardiac ganglion of Homarus, 367.
MAZIA, DANIEL. Chemical and electron microscopic observations on the isolated mitotic apparatus, 355.
MAZIA, DANIEL. *See* SR. FRANCIS SOLANO, 384.
Mechanism of activation and nuclear breakdown in Chaetopterus egg, 87.
Melanin ingestion by macrophages in Xenopus fin, 490.
MERKEL, JOSEPH R. The bacterial reduction of nitro compounds, 391.
Metabolic inhibitors, effects of on in vitro spermatogenesis of Cecropia silkworm, 188.
Metabolic poisons, reactions of amphibian gastrulae to, in vitro, 466.
Metabolism of silkworm during diapause and development, 320.
Metamorphosis, respiratory metabolism of silkworm during, 320.
MEWALDT, L. R. *See* DONALD S. FARNER, 434.
Midpiece of Arbacia sperm, isolation and possible role of, 367.
Mitotic apparatus, chemical and electron microscopic observations of, 355.
Mitotic inhibitor in starfish ovary extracts, properties of, 373.
MIURA, YOSHIAKI. Effect of some antibiotics on the cleavage of Arbacia eggs, 378.
MIURA, Y., AND R. J. NEFF. Qualitative analysis of acid soluble purine and pyrimidine bases in developing Echinarachnius eggs, 379.
MOSER, JOHN C. *See* BERTRAM SACKTOR, 166.
Moulting and growth in Balanus improvisus, 420.
Mucous substances, possible nutritive function of, 381.
Mytilus edulis, particle filtration in, 477.

NADI reaction in echinoderm exogastrulae, 62.
Naid (Aulophorus), regeneration of anterior end of, 80.

NANNEY, DAVID L. Nucleo-cytoplasmic interaction during conjugation in Tetrahymena, 133.
Neanthes lighti, distribution of in Salinas River estuary, in relation to salinity, 335.
NEFF, ROBERT J. Isolation of midpieces from the sperm of Arbacia punctulata and observations on the role of the midpiece in sperm motility, 367.
NEFF, R. J. *See* Y. MIURA, 379.
Neoblasts, role of in regeneration of Aulophorus, 80.
Nereis limbata, surface active material obtained from, 380, 383.
Nerve, amphibian sciatic, structural and in vitro diffusion characteristics of, 358.
Neural crest derivatives in chick, 355.
Neurophysiology of insect chemoreceptors, 257.
Neurula, distribution of pentose nucleic acid in, 348.
Nitro compounds, bacterial reduction of, 391.
Nitrogen, origin and distribution of in Teredo, 316.
NORRIS, PHYLLIS S. *See* F. T. HAXO, 374.
Nuclear breakdown, mechanism of in Chaetopterus egg, 87.
Nucleic acid, correlation of synthesis of with respiration of sea urchin embryos, 412.
Nucleic acid distribution in Arbacia egg, 392.
Nucleic acid, distribution of in gastrula and neurula of Triturus, 348.
Nucleic acid turnover rates in Arbacia gametes, 386.
Nucleo-cytoplasmic interaction during conjugation in Tetrahymena, 133.
Nutritional requirements of Tetrahymena, 269.

OBSERVATIONS on macrophage behavior in fin of Xenopus larva, 490.
OLSON, JOHN M. *See* ARTHUR S. BRILL, 371.
Origin and distribution of nitrogen in Teredo, 316.
ORNSTEIN, NORMA. *See* JOHN R. GREGG, 466.
Osmoregulating capacity in populations occurring in different salinities, 369.
Osmotic properties of marine teleost and elasmobranch erythrocytes, 289.
Osmotic studies of Spisula egg, 382.
OSTERHOUT, W. J. V. Surface active material obtained from Nereis limbata, 380.
OSTERHOUT, W. J. V. Protamin in an extract of the sperm of Nereis limbata, 379.
OSTERHOUT, W. J. V. *See* THEODORE SHEDLOVSKY, 383.
Ovum of Spisula, fertilization and artificial activation of, 213.
Oxidation patterns in echinoderm exogastrulae, 62.
Oxidative systems of Tetrahymena, effects of x-irradiation on, 373.
Oxygen, permeability of silkworm integument to, 308.
Oxygen tension, effect of on in vitro spermatogenesis of Cecropia silkworm, 188.
Oxygen uptake in Phormia larva, 402.

PARPART, ARTHUR K. The motion of echinochrome granules in the unfertilized egg of Arbacia punctulata, 368.
Parthenogenetic activation of Spisula egg, 213.
Parthenogenetic agents, action of on interior protoplasm of Chaetopterus egg, 375.
Particle filtration in ascidians and lamellibranchs, 477.
Patiria miniata, oxidation patterns in exogastrulae of, 62.
Patterns of oxidation in echinoderm exogastrulae, 62.
Pentose nucleic acid, distribution of in gastrula and neurula of Triturus, 348.
Periplaneta, dephosphorylation of ATP by tissues of, 166.
Permeability of the erythrocyte-like cells of Phascolosoma, 128.
Permeability of Phormia integument to oxygen, 402.
Permeability of silkworm integument to oxygen and carbon dioxide, 308.
PETTIBONE, MARIAN H. Fresh-water polychaetous annelid, Manayunkia speciosa, Leidy, from Lake Erie, 149.
Pharmacological studies on marine invertebrates, 387.
Phascolosoma gouldi, permeability of erythrocyte-like cells of, 128.
Phormia larva, cutaneous and tracheal respiration in, 402.
Phormia regina, means of adhesion of puparia of to substrate, 442.
Phormia regina, stimulation of tarsal chemoreceptors of, 257.
Phosphatase activity in adult worker honey bees, 154.
Photoperiodic responses of testes of white-crowned sparrows, 434.
Photosynthesis and phototaxis in Ulva lactuca gametes, 103.
Photosynthetic activity of phycobilans in red and blue-green algae, 374.
Physiological aging in adult worker honey bee, 154.
Physiology of insect diapause, 174, 188, 320.
Pigment, synthesis of during reconstitution in Tubularia, 450.
PIROZYNSKI, W. J. *See* L. VON BERTALANFFY, 240.

Plasmin and thrombin, synthetic substrates for, 360.
Platysamia cecropia, in vitro assay of growth and differentiation hormone of, 174, 188.
Polarity of germ layers in echinoderm embryos, 62.
Polychaete, fresh water, from Lake Erie, 149.
Polychaete, Neanthes, distribution of in relation to salinity in Salinas River estuary, 335.
Potential, membrane, changes in during sodium extrusion and potassium accumulation in frog sartorius muscle, 385.
Potential, membrane, effect of rapid shifts of sodium and potassium on, 368.
Potassium accumulation and sodium secretion in Ulva lactuca, 366.
Potassium and sodium movements in rabbit polymorphonuclear leukocytes, 356.
Process by which puparia of flies become fixed to substrate, 442.
PROSSER, C. L. *See* JOHN D. ANDERSON, 369.
Protamin in an extract of Nereis sperm, 379.
Protein fractions from echinoderm eggs and sperm, 389.
Proteolytic factors in Chaetopterus eggs, 374.
Protozoa, mating types in (Tetrahymena), 269.
Protozoa, nucleo-cytoplasmic interaction during conjugation of, 133.
Puparia, means of attachment of to substrate, 442.
Purine and pyrimidine bases in developing Echinarachnius eggs, 379.
Pyloric caeca of Asterias, structure and function of, 47.

QUAHOG, cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle of, 200.
Quantititive study on regional distribution of pentose nucleic acid in gastrula and neurula of Triturus, 348.

RADIOPHOSPHORUS ingested by Habrobracon females, fate of, 296.
Radiosulfur, effect of on development of Arbacia eggs, 364.
Rana pipiens gastrulae, reactions of to metabolic poisons in vitro, 466.
RAPAPORT, S. *See* I. M. KLOTZ, 377.
Rat, relation between tissue respiration and body size in, 240.
Reactions of gastrulating amphibians to metabolic poisons, 466.
Receptors of amphibian tongue, effects of various acids, on 391.
Reconstitution in Tubularia, synthesis of pigment during, 450.
Red cells of marine teleosts and elasmobranchs, isotonic solutions for, 289.
Redox indicator patterns in relation to echinoderm exogastrulation. I. Oxidation patterns, 62.
Regeneration of anterior end of Aulophorus furcatus, with special reference to effect of x-rays, 80.
Regional distribution of pentose nucleic acid in Triturus gastrulae and neurulae, 348.
Relation of body weight to body surface area in marine fishes, 285.
Respiration of amphibian gastrulae in vitro, as affected by metabolic poisons, 466.
Respiration, cutaneous and tracheal, in Phormia larva, 402.
Respiration rate, correlation of with DNA synthesis in sea urchin embryos, 412.
Respiration of silkworm, 308.
Respiration of tissues, growth and basal metabolism, 240.
Respiration of Ulva gametes, 103.
Respiratory metabolism of silkworm during diapause and development, 320.
Respirometer, new type of, 362.
Ribonuclease activity in rat liver fractions, 359.
Ribonuclease, structure of, 354.
RIESER, PETER, AND ALVIN M. KAYE. Release of an anticoagulant from irradiated Spisula eggs, 380.
ROCKSTEIN, MORRIS. Some aspects of physiological aging in the adult worker honey bee, 154.
Roles of darkness and light in photoperiodic response of testes of white-crowned sparrows, 434.
RONKIN, R. R. Surface activity of hypobranchial mucus of Busycon and possible nutritive function of mucous substances, 381.
RONKIN, R. R. *See* SAMUEL P. MARONEY, JR., 378.
ROSENBERG, ALBURT M. The effect of x-rays on the ability of marine egg protoplasm to undergo two colloidal reactions, 381.
ROSENBERG, E. V. H. *See* I. M. KLOTZ, 377.
ROSENTHAL, HAROLD L. Embryonic development of the lordotic and normal guppy, Lebistes reticulatus (Peters), 160.
ROTH, JAY S. Ribonuclease activity in rat liver fractions, 359.
ROTH, JAY S. *See* HERBERT J. EICHEL, 373.
ROTH, JAY S. *See* J. W. GREEN, 364.

SABELLID annelid from Lake Erie, 149.
SACKTOR, BERTRAM, GERARD M. THOMAS, JOHN C. MOSER AND DONALD I. BLOCH. Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, Periplaneta americana (L.), 166.
Salinity, relation of to distribution of Neanthes in Salinas River estuary, 335.
Salivary glands, role of in attachment of insect puparia to substrate, 442.
Salt marsh Vaucheriae, 395.
Samia walkeri, in vitro assay of growth and differentiation hormone of, 174, 188.
SANDEEN, MURIEL I., GROVER C. STEPHENS AND FRANK A. BROWN, JR. Persistent diurnal rhythms of oxygen consumption in the periwinkle, Littorina littorea and the oyster drill, Urosalpinx cinereus, 381.
SANDEEN, MURIEL I. *See* FRANK A. BROWN JR., 371.
Sarcoma 37 ascites tumor, effect of aqueous extract of beef spleen on cells of, 363.
SCHECHTER, VICTOR. Osmotic and other studies of the Spisula egg, 382.
SCHMIDT, EDMOND L., AND CARROLL M. WILLIAMS. Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture, 174.
SCHNEIDERMAN, HOWARD A., MELVIN KETCHEL AND CARROLL M. WILLIAMS. The physiology of insect diapause. VI. Effects of temperature, oxygen tension and metabolic inhibitors on in vitro spermatogenesis in the Cecropia silkworm, 188.
SCHNEIDERMAN, HOWARD A., AND CARROLL M. WILLIAMS. The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development, 320.
SCHNEIDERMAN, HOWARD A., AND CARROLL M. WILLIAMS. Discontinuous carbon dioxide output by diapausing pupae of the giant silkworm, Platysamia cecropia, 382.
SCHUB, YONYNAH. *See* WALTER L. WILSON, 389.
SCOTT, DWIGHT B. MCNAIR. Observations on the carbohydrate metabolism of the developing Chaetopterus egg, 359.
SCOTT, GEORGE T. *See* HUGH R. HAYWARD, 366.
SCOTT, GEORGE T., AND HUGH R. HAYWARD. The influence of metabolic factors on the distribution of sodium and potassium ions in the green alga, Ulva lactuca, 358.
Sea-lettuce, photosynthesis and phototaxis in gametes of, 103.
Sea urchin embryo, correlation of DNA synthesis with rate of respiration of, 412.
Senescence in honey bee, 154.
Sensory receptors in Laccophilus, 115.
SETON, SR. ELIZABETH. *See* CLARE M. WILBER, 387.
Sexing Arbacia and other marine animals, 365.
Sexual differences in dephosphorylation of ATP by cockroach tissues, 166.
SHANES, A. M. Structural and in vitro diffusion characteristics of intact and "desheathed" sciatic nerves from the toad (Bufo marinus) and from the bullfrog (Rana catesbiana), 358.
SHANKLIN, D. R. Cation interrelationships at the Fundulus ectoderm, 368.
SHANKLIN, D. R. A rare poly-anomalous embryo, 382.
SHAVER, JOHN R. The development of isolated blastomeres of Fundulus heteroclitus, 383.
SHEDLOVSKY, THEODORE, AND W. J. V. OSTERHOUT. Surface active properties of an extract of the sperm of Nereis limbata, 383.
Shell growth in Balanus, 420.
SHERRY, SOL. *See* WALTER TROLL, 360.
Silkworm, respiratory metabolism of during diapause and development, 320.
Silkworm, studies on integument of, 308.
SMITH, RALPH I. The distribution of the polychaete Neanthes lighti in the Salinas River estuary, California, in relation to salinity, 335.
Sodium and potassium ion distribution in Ulva lactuca as affected by metabolic factors, 358.
SOLANO, SR. FRANCIS, AND DANIEL MAZIA. The role of the vitelline membrane in the fragmentation of the Arbacia egg by centrifugal force, 384.
Sparrows, white-crowned, roles of darkness and light in photoperiodic response of testes of, 434.
Spermatogenesis in white-crowned sparrows, 434.
Spermatogonia, use of in in vitro assay of growth and differentiation hormone of Lepidoptera, 174, 188.
Spiracles, role of in respiration of Phormia larva, 402.
Spisula (Mactra) egg, fertilization and artificial activation of, 213.
Squid axon, electrochemical potential difference of chloride ion in, 378.
Starfish, structure and function of pyloric caeca of, 47.
STEARNS, RICHARD N. Alteration in developmental pattern arising from cell membrane

damage caused by elliptically polarized electric currents in Rana pipiens eggs, 385.
STEARNS, RICHARD N. Electric current distribution in a multicellular system as determined by the pattern of biological events resulting from exposure of developing Rana pipiens eggs to externally applied elliptically polarized currents, 384.
STEINBERG, MALCOLM S. Studies on the mechanism of physiological dominance in Tubularia, 360.
STEPHENS, GROVER C. *See* H. MARGUERITE WEBB, 386; MURIEL I. SANDEEN, 381.
STEPHENSON, WILLIAM K. The effect of rapid shifts of sodium and potassium on the membrane potential of muscle fibers of the spider crab, Libinia emarginata, 368.
STEPHENSON, WILLIAM K. Membrane potential changes in fibers of frog sartorius muscle during sodium extrusion and potassium accumulation, 385.
Stimulation of blowfly tarsal chemoreceptors, 257.
STROMINGER, JACK L. *See* HERMAN M. KALCKAR, 391.
Strongylocentrotus purpuratus embryo, correlation of DNA synthesis with respiration of, 412.
Strongylocentrotus purpuratus, oxidation patterns in exogastrulae of, 62.
Structure and function in the pyloric caeca of Asterias forbesi, 47.
Studies on integument of silkworm, VII, 308.
Study of chemoreception in aqueous and gas phases, 115.
Study of isotonic solutions for erythrocytes of marine teleosts and elasmobranchs, 289.
Study of mechanism of activation and nuclear breakdown in Chaetopterus egg, 87.
SULLIVAN, ROBERT L. *See* DANIEL S. GROSCH, 296.
Summation and inhibition following contralateral stimulation of the tarsal chemoreceptors of the blowfly, 257.
Surface area vs. weight in marine fishes, 285.
Surf-clam egg, fertilization and artificial activation of, 213.
Synthesis of DNA, correlation of with respiration of sea urchin embryo, 412.
Synthesis of pigment during reconstitution of Tubularia, 450.

TAKATA, KENZO. Quantitative study of the regional distribution of pentose nucleic acid in the gastrula and neurula of Triturus, 348.
Tarsal chemoreceptors of blowfly, summation and inhibition following contralateral stimulation of, 257.
Taxonomy of Manayunkia speciosa Leidy, 149.
Temperature, effect of on in vitro spermatogenesis of Cecropia silkworm, 188.
Teredo bartschi Clapp, origin and distribution of nitrogen in, 316.
Testes, bird, role of darkness and light in photoperiodic response of, 434.
Testes of Lepidoptera, use of in in vitro assay of growth and differentiation hormone, 174, 188.
Tetrahymena, mating types in, 269.
Tetrahymena, nucleo-cytoplasmic interaction during conjugation of, 133.
Tetrahymena, studies of centrifuged normal and carcinogen-treated, 376.
Thermal inactivation of bacterium-bacteriophage system, 357.
THOMAS, GERARD M. *See* BERTRAM M. SACKTOR, 166.
THRASHER, GEORGE C. A note on specific pigment in the lateral line receptor cell, 386.
Tissue culture, use of in assay of growth and differentiation hormone of Lepidoptera, 174, 188.
Tissues of cockroach, dephosphorylation of ATP by, 166.
Tissue respiration, growth, and basal metabolism, 240.
Toad, macrophage behavior in fin of larva of, 490.
TRKULA, DAVID. *See* MAX A. LAUFFER, 357.
TSUBOI, K. K. Nucleic acid and protein distribution in unfertilized and fertilized eggs, of Arbacia, 392.
Tracheal and cutaneous respiration in Phormia larva, 402.
Triturus pyrrhogaster, distribution of pentose nucleic acid in gastrula and neurula of, 348.
TROLL, WALTER, AND SOL SHERRY. Synthetic substrates for plasmin and thrombin, 360.
Tubularia reconstitution, pigment synthesis during, 450.
Tubularia, studies on mechanism of physiological dominance in, 360.
Type description of Manayunkia speciosa Leidy, 149.

UCA, endogenously regulated diurnal and tidal rhythms in metabolic rate of, 371.
Uca, relationship between time of day and inhibiting influence of low temperature on diurnal chromatophore rhythm of, 386.

Uca, temperature independence of frequency of endogenous tidal rhythmicity of, 371.
Uca, use of autotomized legs in determining phases of tidal rhythm in, 375.
Ultraviolet absorbing substance in marine animals, 391.
Ulva lactuca gametes, photosynthesis and phototaxis in, 103.

VAUCHERIAE from New England salt marshes, 395.
Venus mercenaria, cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle of, 200.
DE VILLAFRANCA, G. W. The apyrase activity of mantle muscle of Loligo pealii, 392.
VILLEE, CLAUDE, DOROTHY VILLEE AND RUTH LAPLACE. Nucleic acid turnover rates in Arbacia eggs and sperm, 386.
VINCENT, W. S. An approach to the problem of DNA constancy, 354.

WALTERS, C. P. *See* M. E. KRAHL, 377.
Warburg measurements of sea urchin embryo metabolism, 412.
WAZETER, F. X. *See* J. W. GREEN, 357.
WEBB, H. MARGUERITE. *See* FRANK A. BROWN, JR., 371.
WEBB, H. MARGUERITE, MIRIAM F. BENNETT, ROBERT C. GRAVES AND GROVER C. STEPHENS. Relationship between time of day and inhibiting influence of low temperature on the diurnal chromatophore rhythm of Uca, 386.
WELSH, JOHN H. *See* SUMNER I. ZACKS, 200.
White-crowned sparrows, role of darkness and light in photoperiodic response of testes of, 434.
WHITELEY, ARTHUR H. *See* JEAN J. COMITA, 412.
WICHTERMAN, RALPH. Studies of the effects of x-radiation upon Paramecium: variations among and within species, 387.
WILBER, CLARE M., AND SR. ELIZABETH SETON. Pharmacological studies on marine invertebrates, 387.
WILLIAMS, CARROLL M. *See* EDMOND L. SCHMIDT, 174; HOWARD A. SCHNEIDERMAN, 188, 320, 382.
WILSON, WALTER L. *See* L. V. HEILBRUNN, 375.
WILSON, WALTER L., AND L. V. HEILBRUNN. The action of cyanide on the protoplasmic colloid, 388.
WILSON, Walter L., AND L. V. HEILBRUNN. Colloidal changes during aging of the Chaetopterus egg, 388.
WILSON, WALTER L., AND YONYNAH SCHUB. The effect of strong centrifugal force on the development of Chaetopterus eggs, 389.
Worker bees, physiological aging in, 154.
Worm, annelid, effect of x-rays on anterior regeneration in, 80.
WRINCH, DOROTHY. A globulite structure for acid insulin sulphate?, 389.
WRINCH, DOROTHY. The structure of ribonuclease, 354.

X-IRRADIATION of Paramecium, 387.
X-rays, effect of on ability of marine egg protoplasm to undergo two colloidal reactions, 381.
X-rays, effect of on anterior regeneration in Aulophorus, 80.
Xenopus laevis, macrophage behavior in fin of, 490.

ZACKS, SUMNER I., AND JOHN H. WELSH. Cholinesterase and lipase in the amoebocytes, intestinal epithelium and heart muscle of the quahog, Venus mercenaria, 200.
ZIMMERMAN, ARTHUR. *See* DOUGLAS MARSLAND, 366.
Zonotrichia leucophrys, roles of darkness and light in photoperiodic response of testes of, 434.

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CONTENTS

Page

BLUM, JOHN L., AND JOHN T. CONOVER
New or noteworthy Vaucheriae from New England salt marshes 395

BUCK, JOHN B., AND MARGARET L. KEISTER
Cutaneous and tracheal respiration in the Phormia larva 402

COMITA, JEAN J., AND ARTHUR H. WHITELEY
The correlation of desoxyribonucleic acid synthesis and the rate of respiration in the sea urchin embryo 412

COSTLOW, JOHN D., JR., AND C. G. BOOKHOUT
Moulting and growth in Balanus improvisus 420

FARNER, DONALD S., L. R. MEWALDT AND STANLEY D. IRVING
The roles of darkness and light in the photoperiodic response of the testes of white-crowned sparrows 434

FRAENKEL, G., AND VICTOR J. BROOKES
The process by which the puparia of many species of flies become fixed to a substrate 442

GOLDMAN, ALLEN S.
Synthesis of pigment during the reconstitution of Tubularia . 450

GREGG, JOHN R., AND NORMA ORNSTEIN
Explant systems and the reactions of gastrulating amphibians to metabolic poisons 466

JØRGENSEN, C. BARKER, AND EDWARD D. GOLDBERG
Particle filtration in some ascidians and lamellibranchs 477

LEHMAN, H. E.
Observations on macrophage behavior in the fin of Xenopus larvae 490